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in Prostate Cancer Progression

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## INTRODUCTION

The progression of prostate cancers from indolent, localized and androgen dependent tumors to aggressive, metastatic and androgen independent tumors involves a series of poorly understood biological and genetic events. Work from a rat prostate cancer model has demonstrated a change in the alternative splicing pathway of fibroblast growth factor receptor 2 (FGFR2) that accompanies this transition. Through a process of mutually exclusive alternative splicing of two exons (IIIb or IIIc) encoding an extracellular domain of FGFR2 gene, two different receptor isoforms (FGFR2-IIIb or FGFR2-IIIc) are produced which differ in their ligand binding specificities. FGFR2-IIIb, expressed in normal prostatic epithelial cells and androgen dependent tumors, is responsive to fibroblast growth factors 7 and 10 (FGF-7 and FGF-10), which are expressed in an androgen dependent manner by prostatic stromal cells and appears to promote epithelial cell differentiation. Androgen independent tumors express FGFR2-IIIc, and it is hypothesized that this change in isoforms may render these cells unresponsive to this pathway of growth regulation. We recently have shown that this transition appears to occur in human prostate cancer cells as well. Since the last report, additional data from human prostate cancer tissue and another animal model have provided additional evidence that loss of the FGFR2-IIIb occurs during cancer progression (Freeman et al., 2003; Naimi et al., 2002). We are investigating the mechanism that leads to this change in alternative splicing of FGFR2 and seek to identify specific protein factors which direct the splicing pathways of this gene in prostate cancer cell lines. The androgen dependent DT3 rat prostate cancer cell line expresses exclusively FGFR2-IIIb and the AT3 cell line is an androgen independent line expressing only FGFR2-IIIc. The report enclosed will outline and summarize our work using a novel genetic screening strategy to identify splicing regulatory proteins involved in maintenance of FGFR2-IIIb expression in prostatic epithelial cells.

## BODY

The body of work demonstrating that a change in the alternative splicing of FGF-R2 may be a key step involved in prostate cancer progression led us to consider strategies towards identifying factors which mediate this splicing choice. It is clear that a loss in expression of FGF-R2 (IIIb) by itself is unlikely to completely explain the development of androgen independent and/or metastatic growth. Nonetheless, the involvement of this receptor in pathways of growth regulation in the prostate suggests that loss of the normal alternative splicing choice for this receptor may be one molecular event in a series of steps in the progression of prostate cancer. This hypothesis is supported by studies using the Dunning rat prostate tumor model showing that some reversal of the more aggressive phenotype can be achieved through restoration of the FGFR2 (IIIb) expression in androgen independent, metastatic AT3 cells. Therefore, we suspect that identification of the protein factors which result in maintenance (or loss) of FGF-R2 regulated splicing may further contribute to our understanding of prostate cancer. Our published studies strongly suggest that a key component of regulation is achieved through the expression of at least one factor in DT3 cells which is required in order to repress exon IIIc splicing and/or activate splicing of exon IIIb (Carstens et al., 1998). While a single factor may not achieve both of these effects, it would appear that the presence of a factor mediating IIIc repression is necessary for regulation to be maintained in DT3 cells to prevent loss of FGF-R2 (IIIb) expression. It is hypothesized that identification of a factor mediating IIIc

repression in DT3 cells will demonstrate a protein whose function or activity is lost in the progression of many prostate cancers, with loss of splicing regulation being one consequence.

**Further characterization of intronic RNA cis-elements facilitates establishment of the genetic screening.** In order to better characterize the RNA cis-elements that are required for exon IIIc repression a number of studies performed in the lab have been necessary to facilitate the Tasks outlined in the proposal. As previously discussed in the proposal, one RNA cis-element referred to as ISAR, is a 20 nucleotide intronic sequence upstream of exon IIIc that is required for repression. In more recent studies, we have shown that the function of ISAR involves formation of an RNA stem structure with another element we refer to as ISE-2 which is located upstream of ISAR in the same intron. Interestingly we found that the primary sequence of ISAR is not required for its role in splicing repression, but that a non-sequence specific RNA stem structure is sufficient to carry out the function of ISAR and ISE-2. These results surprised us and will be important in modifying the constructs to be used in the screens underway to identify specific proteins that mediate exon IIIc repression. These results were reported in *The Journal of Biological Chemistry* (Muh et al., 2002)(See Appendix, note in particular Fig. 5). In more recent studies, we have further analyzed the functions of the regulatory cis-elements contained in intron 8, between exons IIIb and IIIc (see schematic demonstrating these elements in Fig. 1 in Muh et al.). We previously demonstrated that, in DT3 cells, ISAR plays a role both in activation of IIIb splicing as well as in repression of exon IIIc splicing (Carstens et al., 1998). Therefore, these new results suggested that a non-sequence-specific RNA stem structure is required for coordinated activation of IIIb and repression of IIIc, and we have in fact subsequently shown this to be the case (data not shown). However, it was still also clear that this RNA structural element was not sufficient to enforce splicing regulation since deletion of other elements (including ISE-1 and ISE-3) also caused a loss of splicing regulation.

In order to further evaluate the differential contributions and interdependence of the cis-elements in intron 8 we developed a heterologous system in which the functions of these elements could be determined. We obtained splicing constructs from Tom Cooper (Baylor Univ.) that consists of different exon and intron sequences derived from cardiac Troponin I (cTNI) (Cooper, 1998). One construct contained an exon, 33.51, that was inefficiently included in both DT3 and AT3 cells and this exon and its 3' and 5' splice sites were cloned into the intron of adenoviral splicing vector pI-11 (see Fig. 4A, top) to yield pI-XN-33.51 (Fig. 6A). This minigene was transfected into DT3 and AT3 cells, followed by RT-PCR to determine levels of 33.51 exon inclusion versus skipping in spliced mRNA products. As shown in figure 6B and 6C (lane 1), 33.51 exon inclusion was only 3.0% in DT3 cells and 3.4% in AT3 cells. Minigenes were then generated in which FGFR2 intron 8 sequences were positioned immediately downstream of this exon (Fig 6A). The FGFR2 sequences were designated as numerically assigned Intron Fragments (IF). A 614 nt region from the middle of this intron that is dispensable for regulation was not included in any minigenes. As seen in figure 6B (lane 2), a region of FGFR2 intron 8 that began just downstream of the IIIb 5' splice site and includes ISE-1, ISE-2, ISAR, and ISE-3 and consisting of 566 nt (Intron Fragment 1, or IF1) dramatically activated splicing of the upstream 33.51 exon to 28.4 percent in DT3 cells. However, when ISAR was deleted (IF2), exon inclusion was only 2.7 percent which was comparable to the control (Fig. 6B, lane 3). Thus, *cis*-elements in this intron, which include ISE-1, ISE-2, ISAR, and ISE-3, efficiently activate splicing of an upstream heterologous exon specifically in DT3 cells. In order to further define the 3' end of ISE3, a deletion of the 59 nt at the 3' end of IF1 and IF2 was made to generate minigenes containing FGFR2 Intron Fragments 3(IF3) and 4(IF4),

respectively. IF3 effected a 34.9% level of exon 33.51 exon inclusion, which was slightly improved compared to IF1, indicating that the additional sequences in IF1 are not required for splicing regulation (Fig. 6B, lane 4). Again, deletion of ISAR in IF4 abrogated this splicing activation (Fig. 6B, lane 5). In order to confirm that the ability of the 507 nt IF3 fragment to activate splicing results from specific FGFR2 elements and not simply changes in intron size, we placed a 507 nt B-globin intron sequence downstream of exon 33.51. This resulted in only 2.3% exon 33.51 inclusion, comparable to the effect of no insertion (Fig. 5B, lane 6). We next tested whether ISE-3 was required for splicing activation by deleting 144 nt downstream of ISAR in IF5. This intron fragment resulted in only 3.1% 33.51 exon inclusion (Fig. 6B, lane 7). Thus, deletion of ISAR or ISE-3 was equivalent; loss of either element resulted in complete loss of splicing activation. To determine whether ISE-1 was required for cell specific splicing activation the effect of IF6 was determined. This intron sequence resulted in 17.1% exon 33.51 inclusion, whereas further deletion of ISAR in IF7 displayed only 4.9% inclusion (Fig. 6B, lanes 8,9). Although IF6 did not activate splicing as efficiently as IF2 or IF4, these results were highly significant as they clearly indicated that a minimal sequence containing only ISE-2 and ISAR (the dsRNA-SE) and the 144 nucleotide ISE-3 element is necessary and sufficient to mediate cell type specific splicing activation of an upstream exon in DT3 cells. The requirement for the ISE-3 element is further shown with IF8, which only contains the dsRNA-SE (ISE-2 and ISAR) and does not activate splicing. In AT3 cells, no increase in exon 33.51 inclusion was seen with any of these insertions, indicating that the ability of elements that increase exon splicing in DT3 cells represent cell-type specific effects.

The heterologous experiments described above demonstrated clearly that intron 8 elements display DT3 cell-type specific splicing activation of an unrelated exon. We also wished to explore the ability of these same elements to repress the splicing when placed upstream of a heterologous exon. To do this we used a similar troponin derived exon, 35(28) which generally yields a level of splicing inclusion of ~50% in both DT3 and AT3 cells with minigene pI-CX-35(28) (Fig. X). When placed the intron fragment (IF 3) containing ISE-1, ISE-2, ISAR, and ISE3 upstream of this exon, we noted efficient repression of splicing in DT3 cells, but not in AT3 cells (in fact some activation was seen in AT3 cells). When ISAR was deleted, there was a loss of efficient splicing repression. Thus, we were able to show, using this heterologous system that ISAR dependent activation or repression of splicing is primarily position dependent. However, when we deleted ISE-1 (IF5) we noted no significant reduction in the ability of ISE-2, ISAR, and ISE-3 to repress splicing. These findings are to be distinguished from those analyzing activation in which deletion of ISE-1 results in a significant reduction in splicing activation. From these findings we conclude that ISE-1 functions in the activation of exon IIIb splicing, but not in exon IIIc repression. Subsequent experiments have also shown that deletion of ISE3, like for ISAR, results in loss of repression of a downstream exon (data not shown). Deletion of ISAR or ISE-3 likewise results in abrogation of the repression when these sequences are placed upstream of a regulated exon, and no repression is seen when these minigenes are transfected into AT3 cells (data not shown). In results that are being prepared for publication we have further characterized ISE-3 and identified an 85 nt minimal fragment that is sufficient to carry out both splicing activation and repression. We have also found that ISE-3 mutations that impair splicing activation of an upstream exon similarly impair splicing repression of a downstream exon. Thus, together with the double stranded RNA element (the dsRNA-SE; ISE-2 and ISAR) it appears that ISE-3 (but not ISE-1) couples exon IIIb inclusion with exon IIIc exclusion in order to switch the splicing pathway. Collectively, these most recent findings using

heterologous exons have led to a revised model of regulation which is shown in figure X. We propose that DT3 cell-type specific splicing of FGFR2 exons IIIb and IIIc is conferred through what we term a "cell-specific splicing activator/repressor complex" that consists of a multiprotein complex of splicing regulatory proteins that cooperatively bind to the dsRNA-SE and ISE-3. Components of this complex then recruit spliceosomal components to activate splicing of the upstream exon IIIb and repress splicing of the downstream exon IIIc. While other cis-elements, including ISE-1 also play a role in regulation, we propose that the dsRNA-SE/ISE-3 element is the critical "switch" that establishes the cell-specific mechanisms in DT3 cells that result in splicing that yields FGFR2-IIIb. We also hypothesize that an epithelial cell-specific splicing regulatory protein is one component of the complex that forms on these elements that leads to function of this complex only in cells that express FGFR2-IIIb.

**Generation of fluorescent reporter minigenes that indicate function of the "cell-specific splicing activator/repressor complex" in DT3 cells.** The results described in the previous section have played an essential role in furthering the progress of this proposal by leading directly to more robust fluorescence based methods of identifying cell-type specific splicing pathways mediated by FGFR2 cis-elements. In the original proposal, we described the generation of a minigene, pEGFP-IIIc, which we planned to use in a genetic screen to identify DT3 cell factors that mediate exon IIIc repression (Fig 2 in original proposal). In this minigenes, an intron was directly introduced into the open reading frame (ORF) of EGFP. Subsequently, FGFR2 intron sequences containing exon IIIc and flanking intron sequences were also included in this intron. Our expectation was that in DT3 cells exon IIIc would be inefficiently included, whereas in AT3 cells exon IIIc would be included and thus GFP activity abolished. While we continued to observe complete skipping of exon IIIc in DT3 cells as expected, we only observed low levels of exon IIIc inclusion in AT3 cells. While this resulted in differential splicing of exon IIIc that could be distinguished by RT-PCR, the differences in EGFP expression that could be detected by flow cytometry were minimal. Additional modifications of this design were not successful in yielding meaningful differences in EGFP activity; differences

The failure of reporter minigenes containing an intron within the EGFP ORF led us to pursue alternative strategies in which either exon IIIb activation or exon IIIc repression could be determined using fluorescence. A large number of different strategies were attempted using different fluorescent reporter genes, different heterologous introns and exons containing FGFR2 cis-elements, and different vector backbones. While it would be excessive to catalog all of the various approaches that we attempted, it is worth pointing out that given the need to carry out stable sets of transfections before the success of any given approach can be assessed, considerable time during the funding period was consumed in this process. As such the timeline of these experiments will be extended beyond the period of funding on this grant. Nonetheless, we now have developed a series of minigenes that permit exquisite fluorescence-based recognition of cell-type specific splicing pathways mediated by FGFR2 cis-elements. Using these minigenes we have established stably transfected clonal cell lines expressing these minigenes. A key aspect of these minigenes that differs from the original minigenes design is that the cDNA sequence encoding the fluorescent protein is located 3' from upstream sequences that contain alternatively spliced regions of the minigenes. Depending on the specific design employed, a specific splicing pathway will yield fluorescence only if the spliced products direct in frame translation of the fluorescent protein. The overall design that was most successful is shown in Fig. 4A and will be briefly described. First, we found that in order to optimize the isolation of stable clones carrying these minigenes that use of an ECMV Internal Ribosome

Entry Site (IRES) downstream of the minigenes and directing translation of the drug resistance for selection was an essential feature. This bicistronic design directs translation of both the minigene and drug selection (neomycin or puromycin resistance) from the same RNA; the 5' ATG start codon directs translation of the minigenes and a second ATG directs translation of the drug resistance gene downstream of the IRES. Therefore, essentially all drug resistant cells express the minigenes placed in the construct. Furthermore, because selection is directly coupled to expression of the minigenes, we also were able to enhance isolation of high expressing clones by increasing the concentration of selective reagent (G418 or puromycin). At the 5' end of the minigenes, we placed an open reading frame encoding a C-terminal region from Protein Kinase C-gamma subunit (PKC) with an optimal Kozak consensus sequence surrounding the 5' ATG. Between this ORF and the ORF encoding the fluorescent protein we inserted an adenoviral intron with optimal 5' and 3' splice sites (the same intron as used in the construct shown in Fig. 1). Two versions of minigenes contained different linker sequences between the 3' splice site and the start of the fluorescent protein ORF. Versions labeled with "pos" contained an additional nucleotide (+1) that was necessary to maintain the same reading frame after splicing such that the fluorescent protein would be translated as a C-terminal fusion with the PKC ORF. Versions labeled "neg" do not contain this extra nucleotide and instead contain an in-frame stop codon after splicing such that a fluorescent fusion protein will not be translated. Both versions contain in-frame stop codons in the intron such that unspliced minigenes will not direct translated of the fluorescent protein. We used cDNAs for two different fluorescent proteins, enhanced green fluorescent protein (EGFP) and monomeric red fluorescent protein 1 (mRFP) which have different excitation and emission optima that permit independent recognition of each protein by flow cytometric analysis. This monomeric red fluorescent protein was recently described as a new variant that more readily yields fluorescence when fused with protein sequences than previous generation red fluorescent proteins (Campbell et al., 2002). In fact, we had found other red fluorescent proteins did not fluoresce when used in our fusion minigenes (data not shown). The rationale for expanding to use of two different fluorescent proteins will be described below.

To set up a system to determine cell type specific splicing by the intron 8 cis-elements, we then placed the 33.51 exon with the different intron fragments from FGFR2 located downstream into the intron of either pPKC-neg-EGFP or pPKC-neg-mRFP. Because exon 33.51 is 46 nt, it adds one nucleotide to the sequence between the PKC ORF and that of the fluorescent protein and thus when included in the spliced mRNA restores the frame that generates a fluorescent fusion protein (Fig. 4B). Thus we predicted that as the percentage of exon 33.51 inclusion increases in response to intron 8 cis-elements, we should see a corresponding increase in fluorescence. Therefore, those intron fragments that increase exon 33.51 inclusion should yield fluorescence in DT3, but not AT3 cells. By flow cytometric analysis, this indeed proved to be the case. As shown in Fig. 4C and 4D, the intron fragments that yielded the best level of exon inclusion (see Fig. 1) could be shown to yield a correspondingly higher level of EGFP fluorescence. In particular, note that the fluorescence was exquisitely sensitive to the presence of ISE3 (IF5 and IF 8) as both intron fragments in which it was absent, even in the presence of ISAR, did not yield significant 33.51 inclusion and therefore displayed no significant level of fluorescence. On the other hand, we did note some increase in fluorescence in the presence of ISE1, but again, this element was not necessary to observe cell type specific exon 33.51 splicing activation. In Fig. 4E, we were able to show that indeed in the context of these minigenes, the level of exon 33.51 inclusion did, in fact correlate well with the percentage of exon 33.51 inclusion. In AT3 cells we noted no significant activation of exon 33.51 inclusion as determined



by fluorescence (Fig. 5) or by RT-PCR (data not shown). Finally, the same minigenes were also made with mRFP instead of EGFP. Rather than present all of the data, in Fig. 6 we simplify the results to show that here also IF3 activates exon 33.51 splicing in DT3, but not AT3 cells, and this can be assessed by red fluorescence.

**Combinations of fluorescence based minigenes with EGFP and mRFP and with different selectable markers permits robust expression cloning with retroviral cDNA libraries.** Using the minigenes we described, we have isolated single cell clones of both DT3 and AT3 cells in which minigenes containing intron fragment 6 are expressed. We have established both EGFP and mRFP based clones and as expected the DT3 clones display fluorescence, but not the AT3 cells. We have now also generated minigenes in which the IRES directs resistance to puromycin. Using these minigenes we have double selected and isolated clones in which two minigenes are stably expressed. One minigene contains EGFP and neomycin resistance and the other minigene contains mRFP with puromycin resistance. This has yielded DT3 cells that have both green and red fluorescence in response to cell-type specific activation of exon 33.51 splicing by IF6. AT3 cells also express both minigenes but do not display significant green or red fluorescence. We plan to screen both cell lines. AT3 cells, if they express a cDNA derived from DT3 cells that is sufficient to activate exon 33.51 splicing are predicted to acquire both green and red fluorescence. Because we can independently sort cells for EGFP or mRFP, having dual validation of changes in fluorescence in response to splicing greatly enhances the chances of success and elimination of background changes in either marker alone. We will also see if overexpression of a single factor in DT3 cells can increase the level of fluorescence. Since the percentage of exon 33.51 is intermediate in these minigenes, it is possible that a single factor, perhaps one that interacts with ISE-3, may be sufficient to further increase the level of inclusion and, therefore, fluorescence. Finally, while this system uses splicing activation to identify factors that regulate FGFR2 splicing, because ISE-3 has been shown to mediate activation of the upstream exon while at the same time repressing downstream exons, the screen is expected to yield exon IIIc repressors as originally planned. Also, by eliminating ISE-1 from the screen, we improve the likelihood that factors identified are specifically involved in the "activator/repressor complex" as shown in Fig. 3.

I will now summarize briefly, the steps made in the achievement of the outlined Tasks.

**Task 1. Set up and perform a genetic screen to identify proteins involved in alternative splicing of FGF-R2.**

- **Use PCR to create an intron within the coding sequence of pEGFP-N1 which disrupts the open reading frame and test this construct for splicing in DT3 and AT3 cells using transfection (months 1-4).**

As described above, we have altered the approach and instead use minigenes that generate N-terminal fusion proteins with fluorescence proteins in response to cell-type specific splicing programs. We have generated both EGFP and mRFP based versions of these minigenes.

- **Introduce FGF-R2 minigene sequences containing exon IIIc into the artificial intron of pEGFP-N1 to obtain plasmid pEGFP-IIIc (months 4-5).**

We have substituted heterologous exons that nonetheless display cell-type specific splicing and

fluorescence using FGFR2 intronic splicing regulatory elements.

- **Transfect DT3 and AT3 cells with pEGFP and confirm efficient exon IIIc splicing in AT3 and repression in DT3. Prepare DT3 cDNA library in mammalian expression vector (months 5-7).**

See above. Briefly, we have transfected minigenes into DT3 and AT3 cells in which exon IIIc repressing elements can be directly screened functionally by fluorescence-based analysis. Regarding the cDNA library, we have opted to use a retroviral cDNA library derived from DT3 cells. We prepared polyA RNA from DT3 cells and had a custom library made through custom cDNA library service from Invitrogen. They provided us with a high quality cDNA library in an entry vector. We used recombination approaches (the Gateway site specific recombination system, Invitrogen) to transfer the library into the pMX retroviral vector (Onishi et al., 1996). We then amplified and analyzed the cDNA library in the retroviral vector. We have confirmed that we have >90% of the vector containing cDNA inserts with an average insert size of approximately 2 kb. Thus a high quality retroviral library is prepared for the screening.

- **Set up series of transfections with pEGFP-IIIc in AT3 cells, screen with DT3 cDNA library and sort cells by FACS analysis (months 7-18).**

We have begun the screening approach. However, as noted above one key development has been that the technique of gene transfer we will use is with a retroviral system rather than transfection. The advantage of such a system is that essentially 100% of cells can be transduced to accept cDNAs and the cDNAs can be selected for continued expression of these cDNAs which will facilitate cloning. The library is ready and we have a packaging and delivery system optimized to infect the cDNA library into clones of cells carrying the fluorescence based minigenes.

- **Isolate cDNAs from fluorescent FACS sorted AT3 cells and sequence the cDNAs. Perform Northern blot analysis in AT3 and DT3 cells to look at differences in expression. (months 18-24).**

This step is imminent. Sorting of retrovirally infected cells should be preformed with the coming 1-2 months.

**Task 2. Rescreen candidate cDNAs to confirm role in splicing repression and examine effects on splicing of endogenous FGF-R2 *in vivo*.**

- **Transfect AT3 cells expressing pEGFP-IIIc with candidate cDNAs to confirm repression of exon IIIc splicing (months 20-24).**
- **Test cDNAs for specificity of splicing repression using FGF-R2 sequences which lack ISAR and using constructs with heterologous exons (months 22-28).**
- **Test cDNAs for ability to activate exon IIIb splicing and to change splicing of the endogenous FGF-R2 gene in AT3 cells from use of exon IIIc to IIIb (months 24-30).**
- **Analyze the effects of transfection of DT3 cell derived cDNAs on AT3 cell growth,**

**including restoration of FGF-7 dependent growth inhibition (months 30-36).**

Because candidate cDNAs have not been isolated, we have been unable to proceed to this step by this date. However, with the much improved, more robust system we have developed, we expect to identify cDNAs soon. I also would also like to point out that the power of the system we have established will make it applicable to other systems of splicing regulation.

## **KEY RESEARCH ACCOMPLISHMENTS**

- Determination that ISE-2 and ISAR form an RNA stem structure that participates in both exon IIIb splicing activation and exon IIIc splicing repression. This stem structure does not contain any specific RNA sequences that are required for either function.
- Characterization of ISE-3, a new RNA cis-element that also required for both IIIb activation and IIIc repression.
- Generation of EGFP and RFP reporter constructs that demonstrate cell-type specific splicing regulation that can be identified through flow cytometric analysis.
- Establishment of single cell clone stably expressing dual fluorescence (EGFP and mRFP) in response to ISE-3 dependent splicing regulation in DT3 cells. AT3 cells carrying the same minigenes that do not fluoresce have also been isolated.
- Preparation of a high quality DT3 cDNA library in a high titer MMLV based retroviral expression vector, pMX.
- Establishment of a high efficiency retroviral packaging system for infection of target cell lines with the DT3 cell derived cDNA library.

## **REPORTABLE OUTCOMES**

### **Abstracts:**

#### **Intronic FGFR2 cis-elements confer cell-type specific splicing regulation to heterologous exons in vivo and in vitro**

*Ruben Hovhannisyan, Stephanie J. Muh, and Russ P. Carstens.*

#### **A novel genetic screen for protein factors that regulate fibroblast growth factor receptor 2 (FGFR2) splicing**

*Stephanie J. Muh, Ruben Hovhannisyan, and Russ P. Carstens.*

Both abstracts presented as posters at the RNA Society Meeting held in Madison, WI May, 28-June 2, 2002.

#### **EGFP based splicing reporter minigenes that permit functional cloning of FGFR2 splicing regulatory proteins**

*Ruben Hovhannisyan, Lori Gostomski, and Russ P. Carstens.*

## **Identification of ISE3, a *cis*-Acting Intronic Element Required for Cell Type Specific Splicing of Fibroblast Growth Factor Receptor 2 (FGFR2)**

*Ruben H. Hovhannisyan and Russ P. Carstens*

Both abstract presented as posters at the RNA society Meeting held in Vienna, Austria, July 1-6, 2003.

### **Publications:**

Muh, S. J., Hovhannisyan, R., and Carstens, R. P. (2002). A non-sequence-specific double stranded RNA structural element regulates splicing of two mutually exclusive exons of fibroblast growth factor receptor 2 (FGFR2). *J Biol Chem* 277, 21.

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### **CONCLUSIONS**

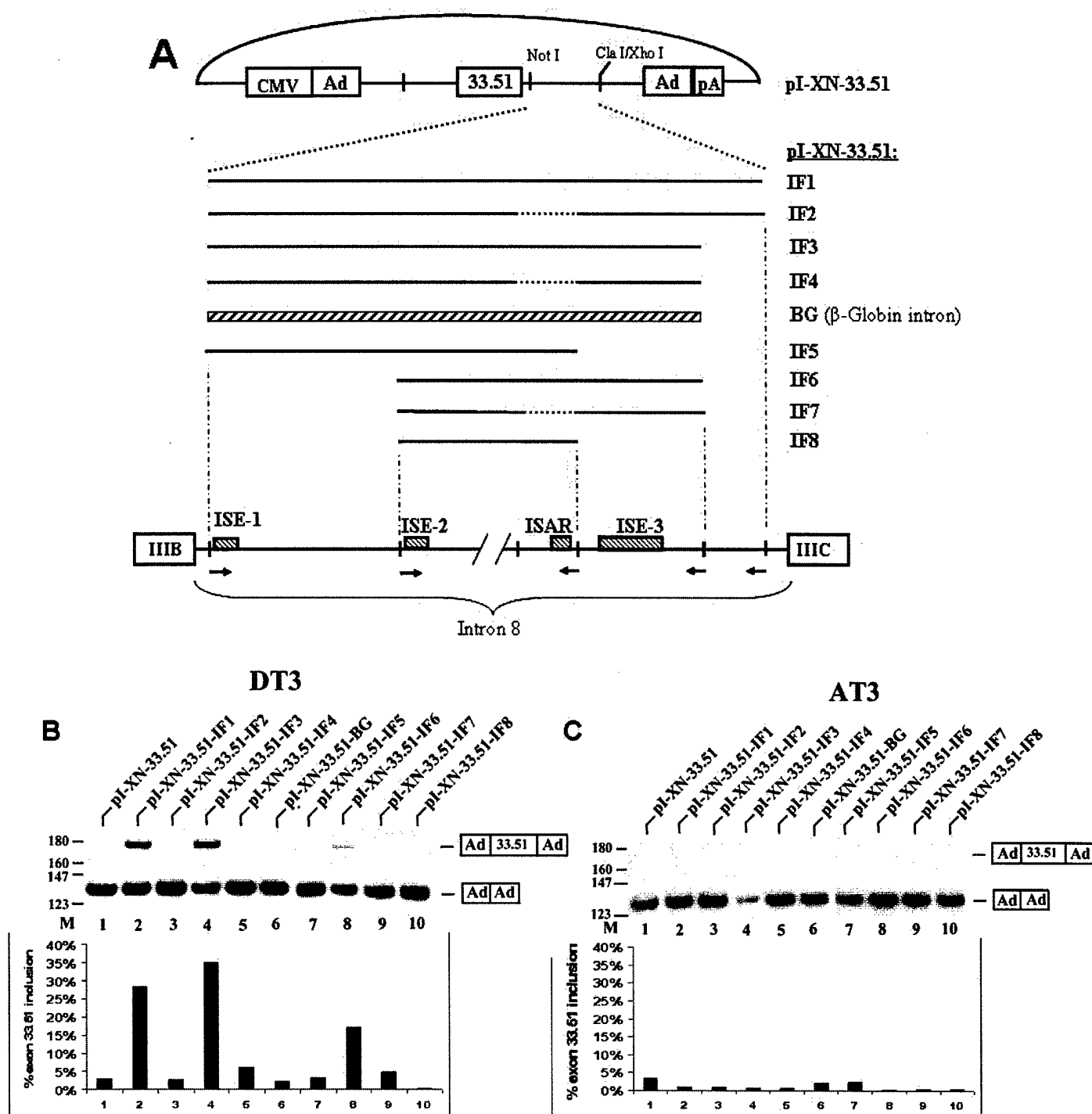
Identification of molecular mechanisms and genes whose expression is involved in prostate cancer progression are crucial for progress in generating future therapies and disease markers. Loss of factors that maintain the epithelial specific FGFR2 (IIIb) isoform and that lead to splicing of the mutually exclusive exon IIIc are hypothesized to be one step in the acquisition of androgen independent growth by aggressive prostate cancers. We are continuing work to identify such factors using a novel genetic screening methodology. Thus far we have been able to show that expression of EGFP or mRFP can be easily assayed by flow cytometry in response to cell type specific splicing pathways and are carrying out screens for cDNAs encoding splicing regulatory factors. Several modifications of the original approach were required in order to make the screen more robust. Significant progress has also been made to identify with greater precision the FGFR2 intronic sequence elements that mediate exon IIIc repression and this information has allowed us to further tailor our methods to identify the factors that carry out splicing repression. We have at present established a very efficient system to use fluorescence to identify FGFR2 splicing regulatory proteins. While we have not completed all of the experiments originally proposed, we have achieved other results that also shed light into FGFR2 splicing regulation. I also believe that the system we have established may be perhaps the most robust and faithful fluorescence based system for identification of splicing regulator to date.

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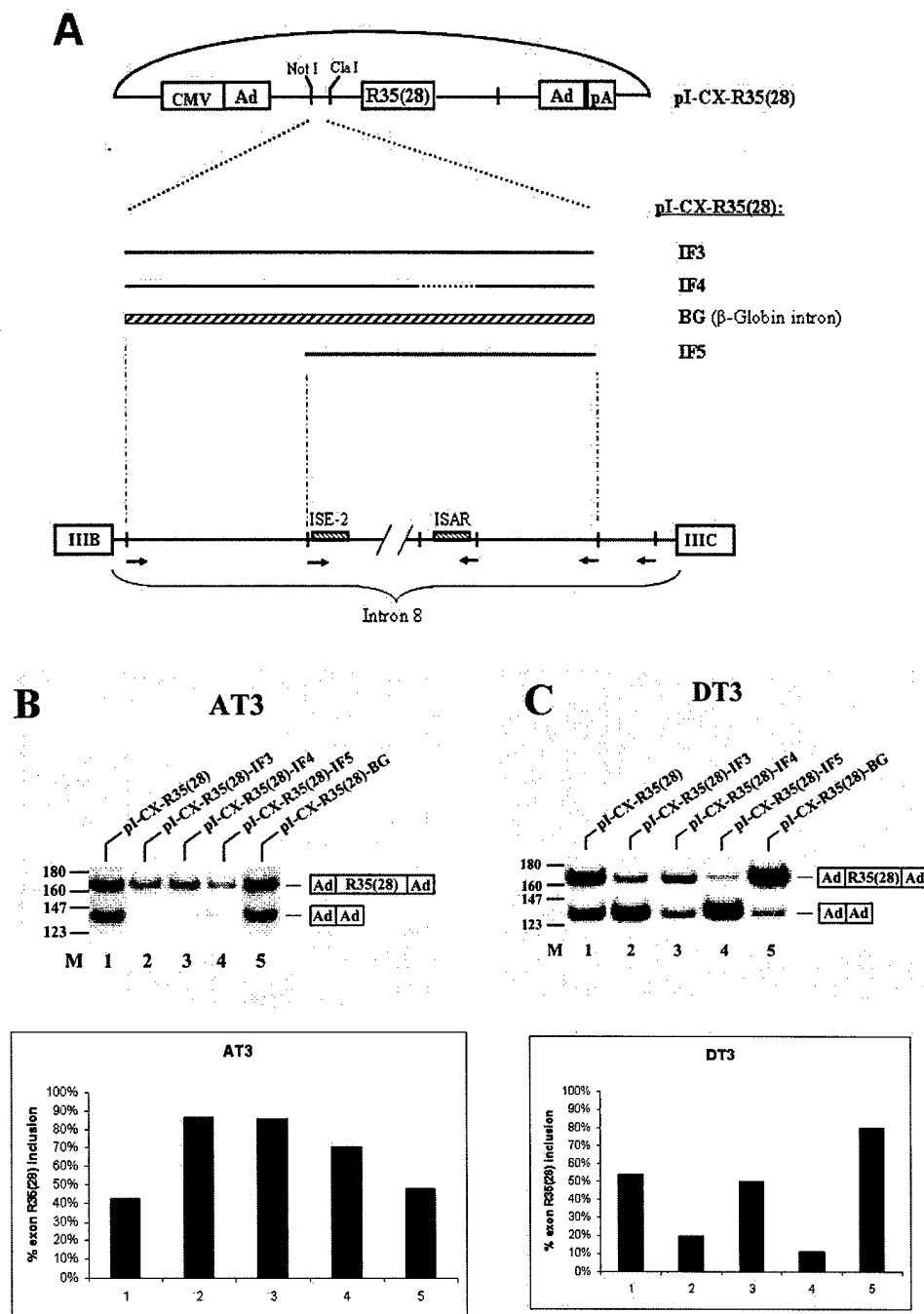
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## APPENDIX:

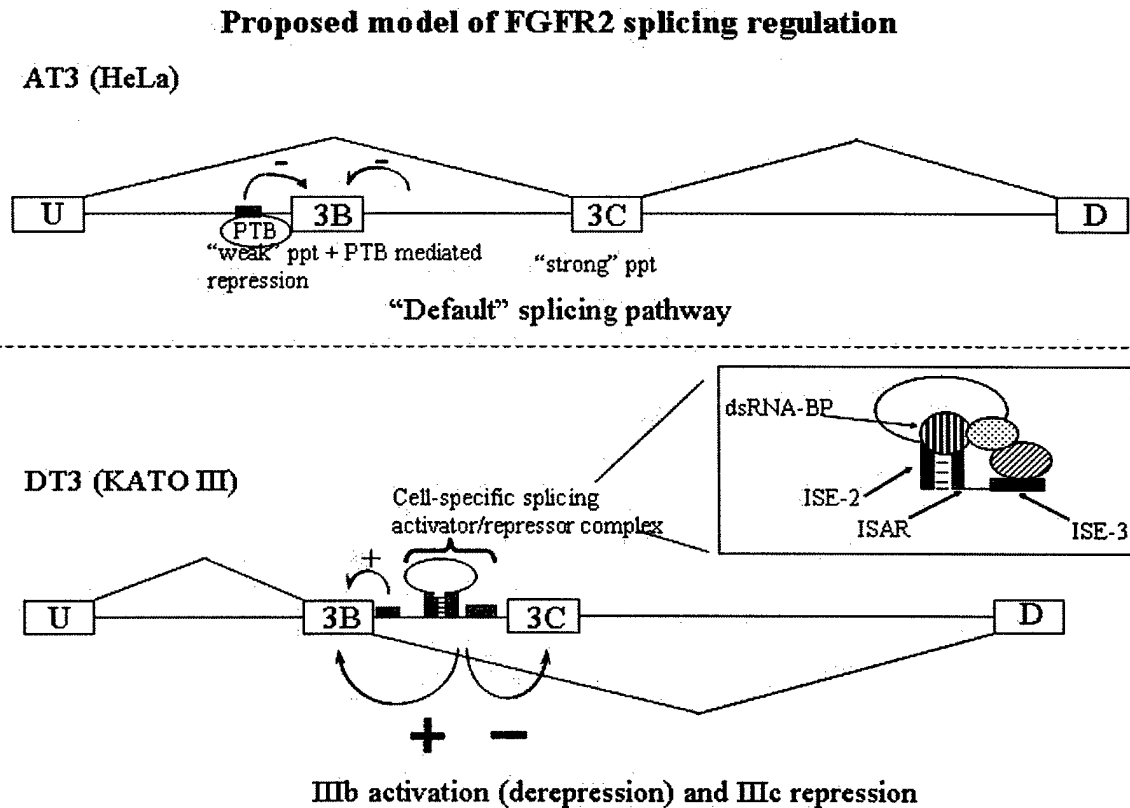
See attached copy of the above Paper from *The Journal of Biological Chemistry*.



**Figure 1.** Intron 8 sequences from FGF-R2 can activate splicing of a heterologous cTroponin I exon located upstream in DT3, but not AT3 cells. **A.** Schematic of pI-XN-33.51 and intron 8 elements from FGF-R2 that are positioned downstream of exon 33.51. Solid lines represent fragments (IF) of this intron that were relocated downstream of the cTNI exon. Dashed lines indicate deleted sequence and the hatched box represents a B-Globin intron sequence. At the bottom is a representation of intron 8 to illustrate the derivation of the intron fragments. Open boxes indicate the exons and arrows indicate positions of PCR primers used to generate the intron fragments. **B.** RT-PCR of DT3 cells stably transfected with these minigenes and graphical representation of % exon inclusion, with positions of the RT-PCR products with inclusion or skipping of the cTNI exon indicated at the right. **C.** Results of transfecting the same plasmids in AT3 cells.



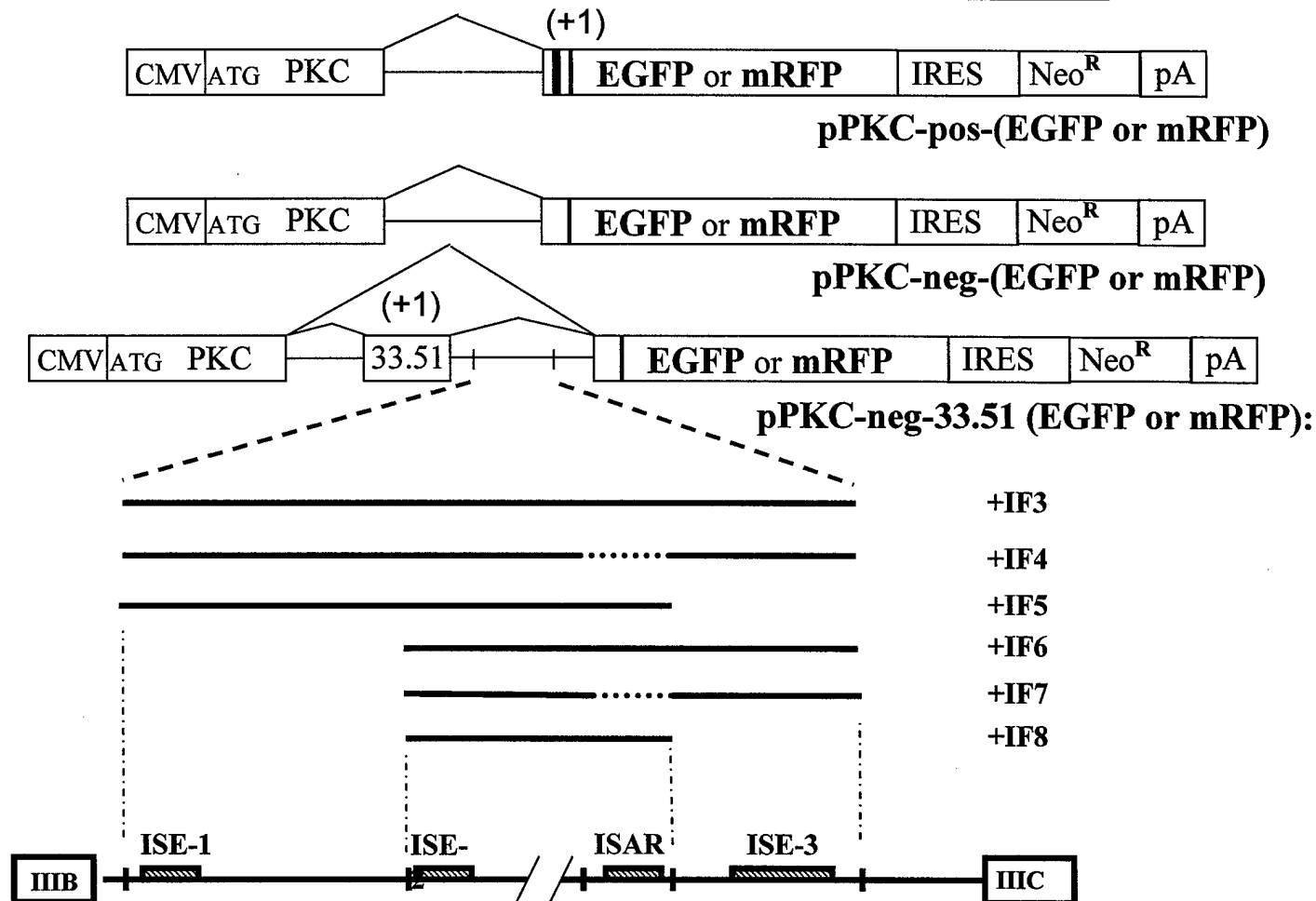
**Figure 2.** Intron 8 sequences from FGF-R2 can repress splicing of heterologous cTroponin I exon located downstream in DT3, but not AT3 cells. **A.** Schematic representation of intron 8 elements from FGF-R2 and numbered constructs and solid lines represent portions of this intron that were relocated upstream of the cTNI (33.51) exon. IF is Intron Fragment from FGF-R2 intron 8. BG is an intronic sequence from rat  $\beta$ -Globin used as a control to confirm the sequence specific nature of the FGF-R2 sequences on splicing. Dashed lines indicate deleted sequence. **B.** RT-PCR of RNA from DT3 and AT3 cells stably transfected with these minigenes. **C.** Graphical representation of percent exon inclusion. Boxes labeled Ad indicate the adenoviral exons and cTNI exon is labeled as 33.51, with positions of the RT-PCR products with inclusion or skipping of the cTNI exon indicated at the right.



**Figure 3.** Schematic representation of a model to account for cell-type specific splicing of FGFR2 mutually exclusive exons IIIb and IIIc. Based on the ability of an intronic fragment containing ISE-2, ISAR, and ISE-3 to confer cell specific splicing activation (upstream) and repression (downstream) to a heterologous exon it is proposed that a multiprotein complex forms on these elements specifically in cells that yield FGFR2-IIIb. ISE-2 and ISAR function requires formation of a double-stranded RNA stem, suggesting a role for a double stranded RNA binding protein in cell-specific regulation. Additional proteins that bind to ISE-3 and or proteins that promote cooperative binding to this intronic region are also indicated.

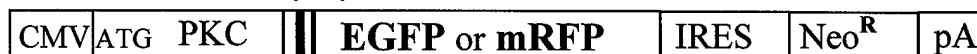


**Fig. 4A** EGFP and mRFP fusion reporter minigenes



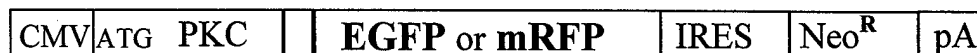
**Fig. 4B**  
Predicted splice products of FGFR2 Minigenes

**pPKC-pos-(EGFP or mRFP): (+1)**



**Generates in-frame PKC-EGFP (or mRFP) fusion protein; Positive fluorescence**

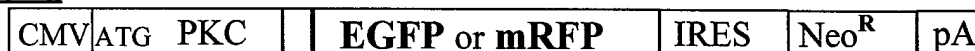
**pPKC-neg-(EGFP or mRFP):**



**Generates out-of-frame PKC-EGFP (or mRFP) fusion protein; No fluorescence**

**pPKC-neg-33.51 (EGFP or mRFP):**

Exon 33.51 skipping

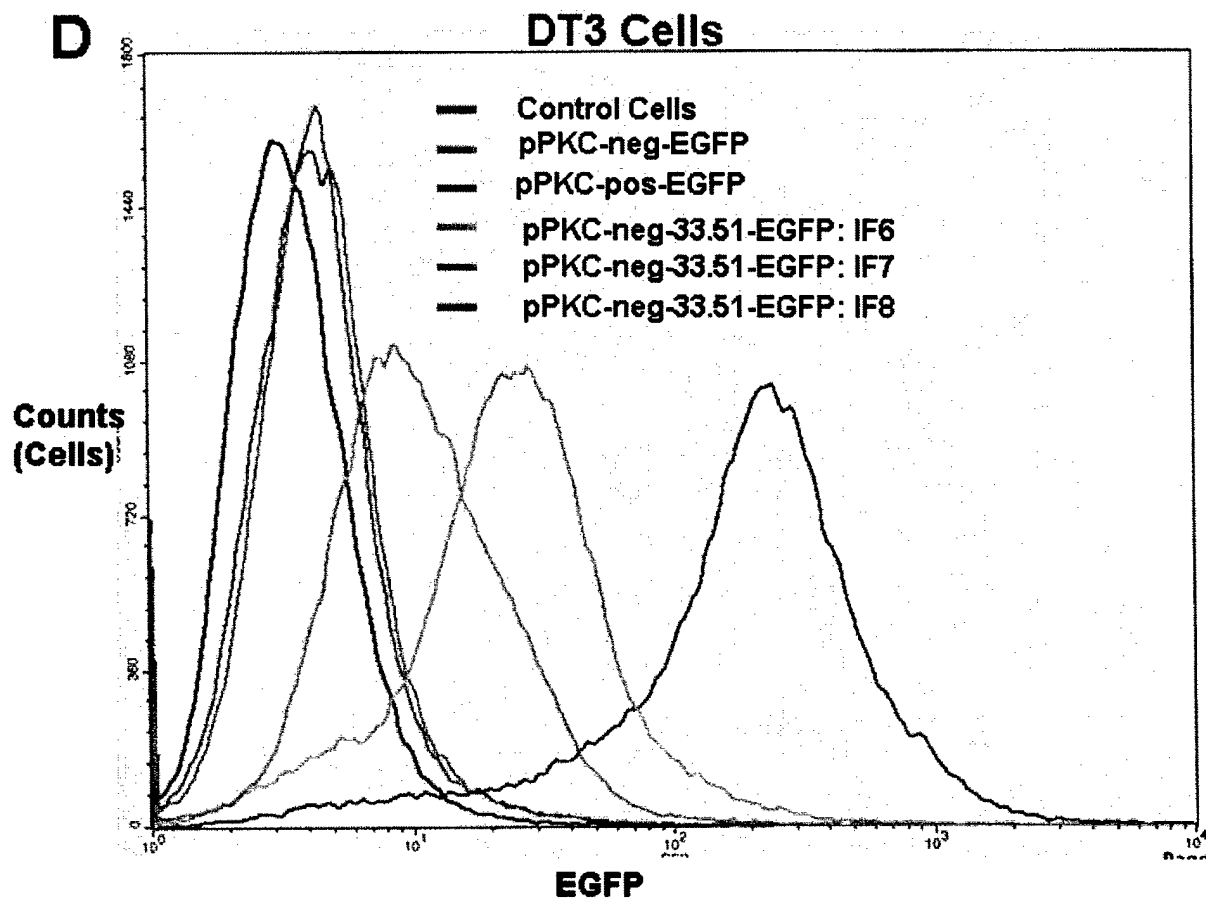
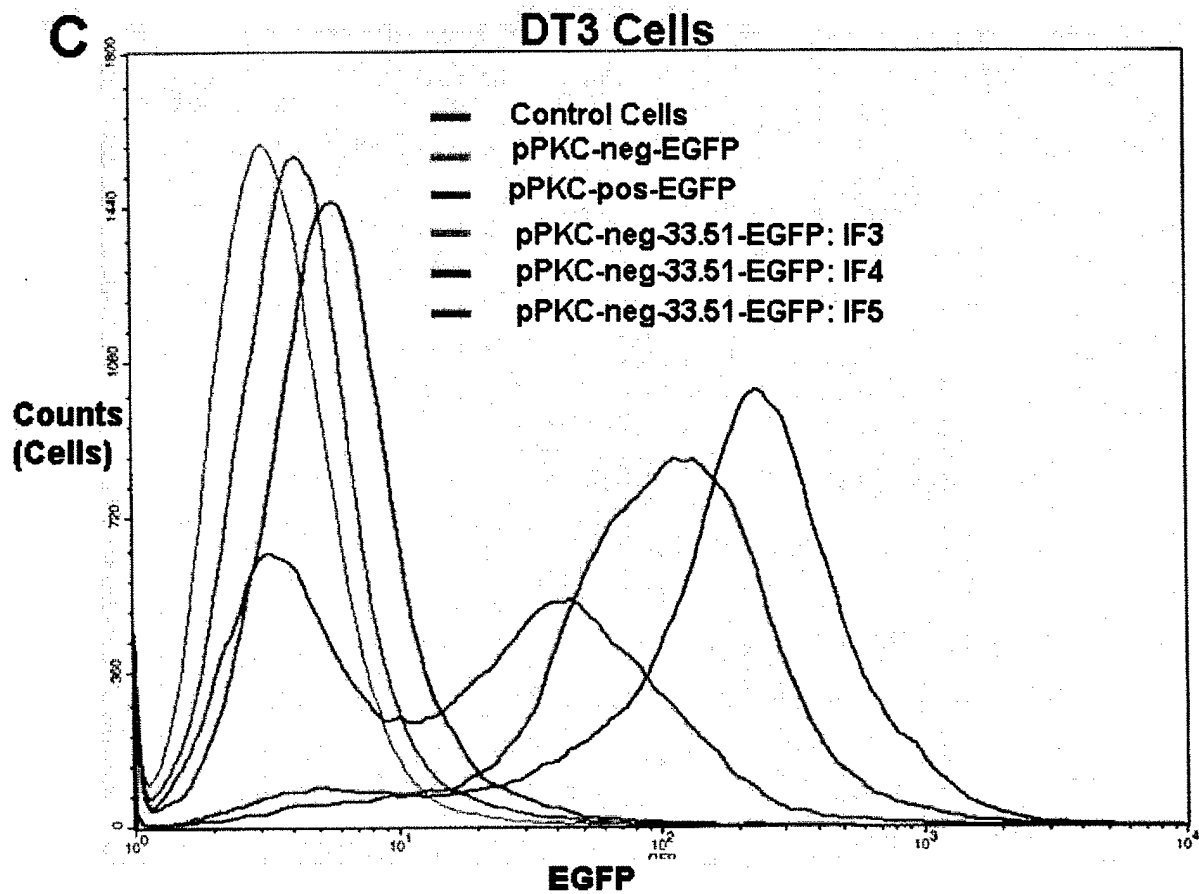


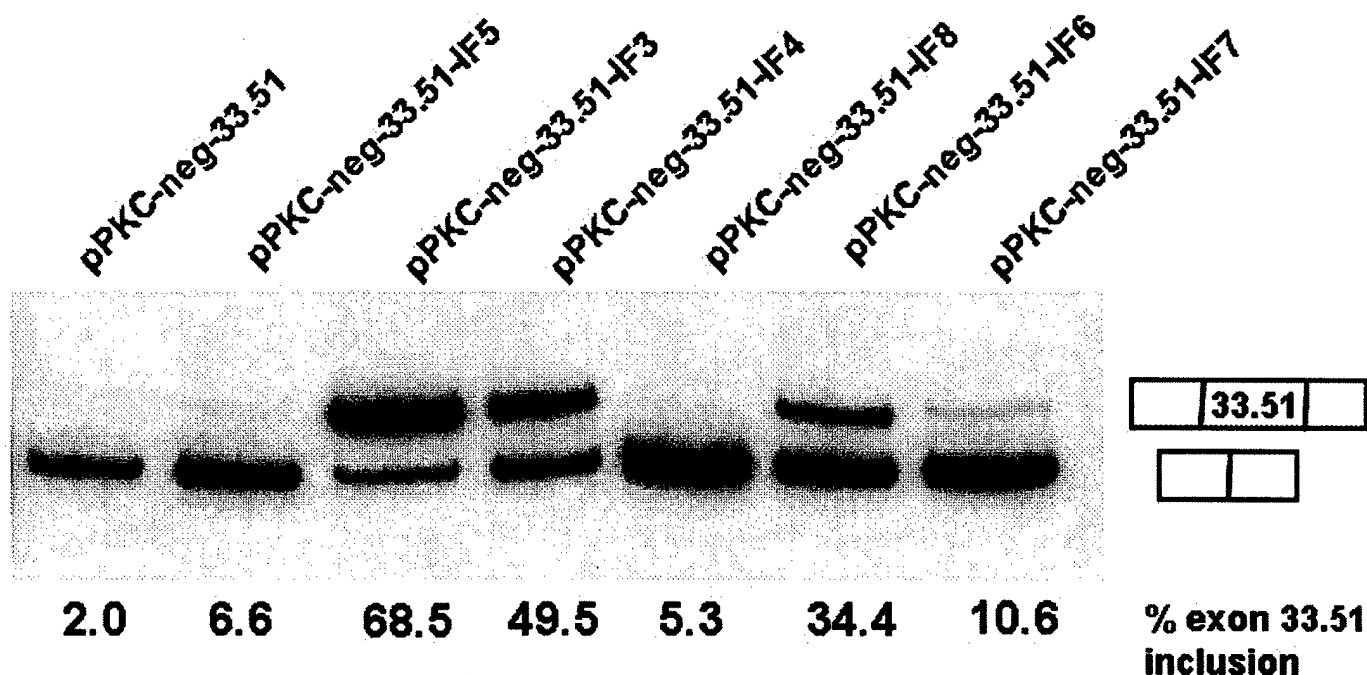
**Generates out-of-frame PKC-EGFP (or mRFP) fusion protein; No fluorescence**

Exon 33.51 inclusion

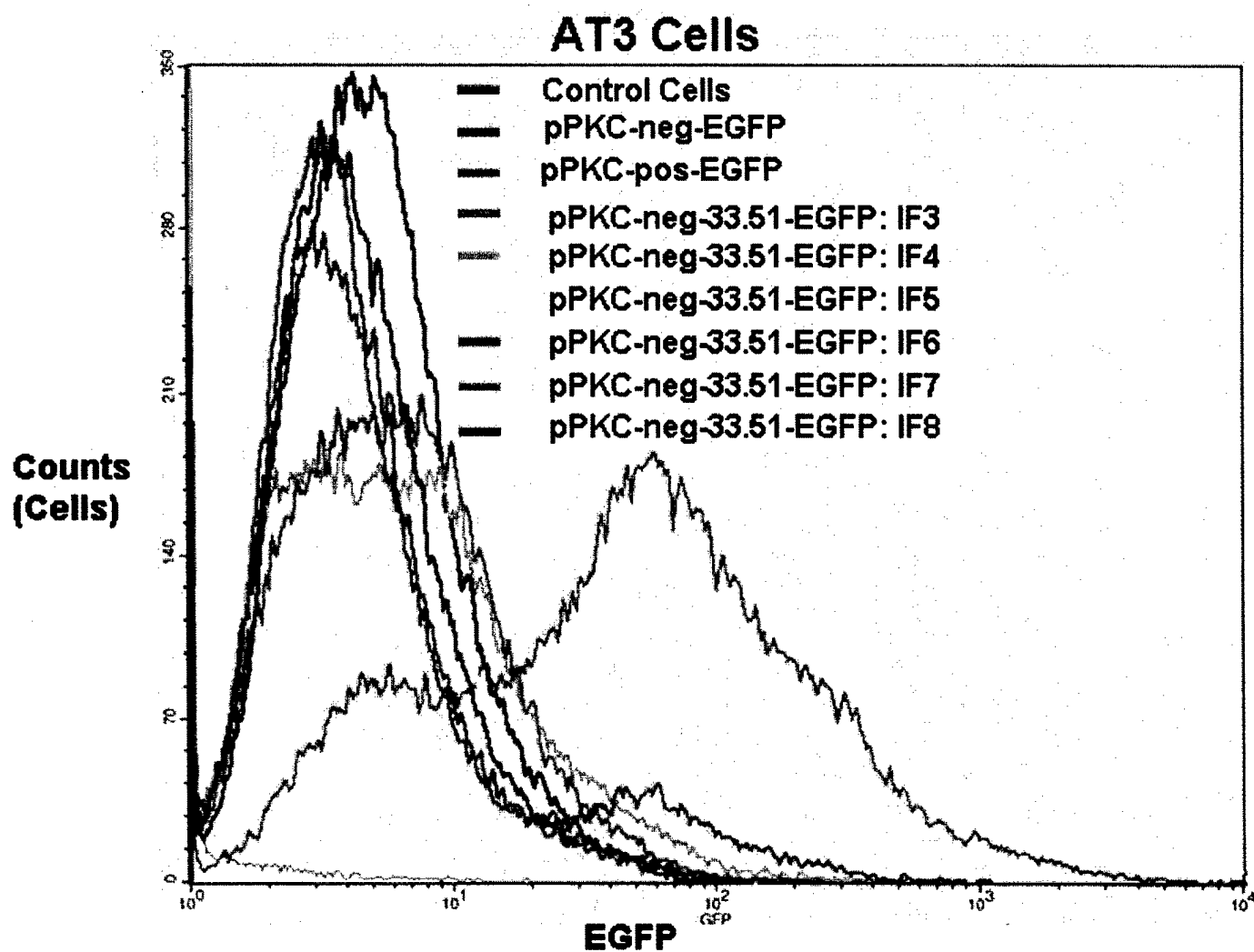


**Generates in-frame PKC-33.51-EGFP (or mRFP) fusion protein; Positive fluorescence**

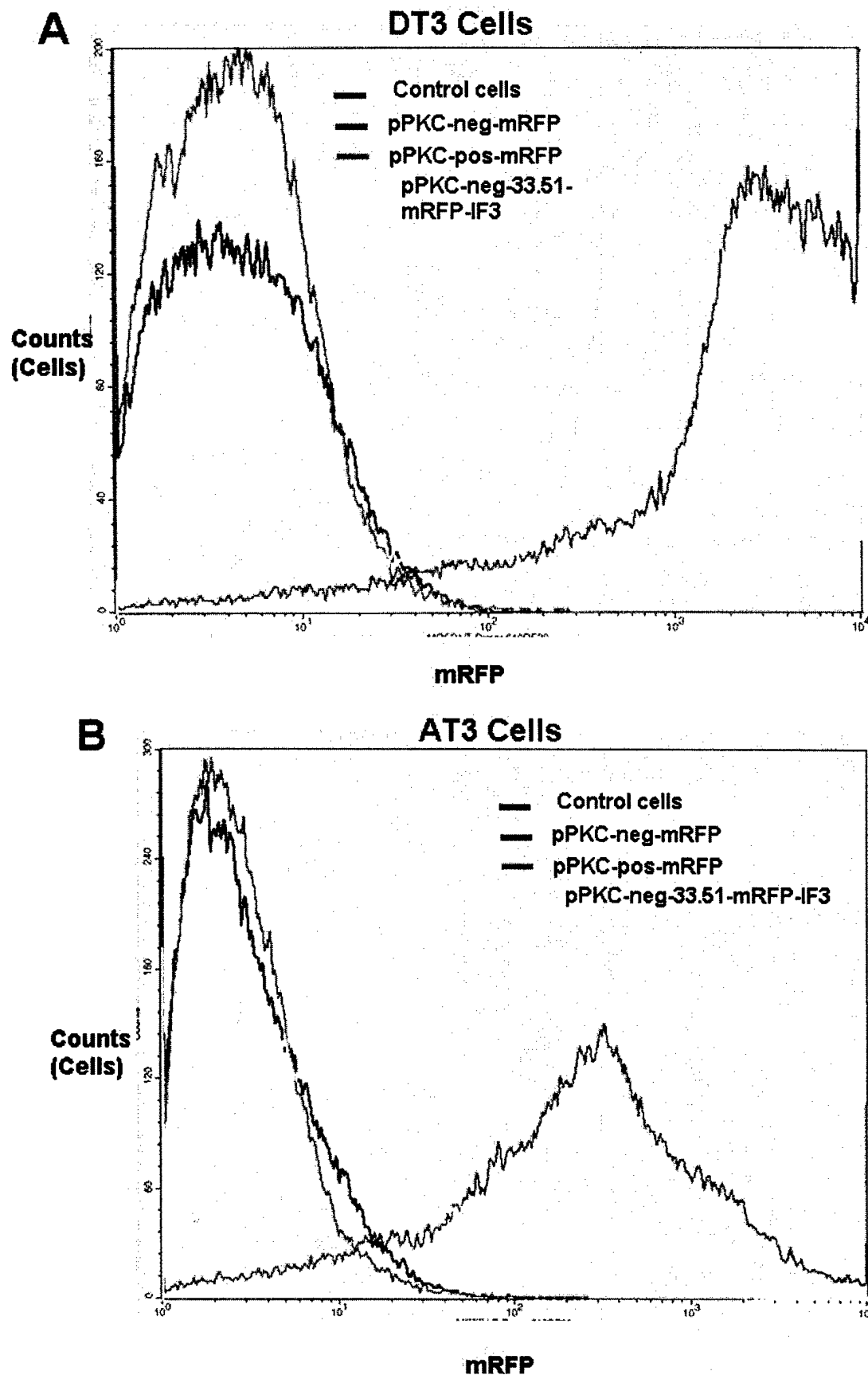


**E**

**Figure 4.** Heterologous Minigenes demonstrate cell-type specific activation of exon 33.51 splicing that is ISE3 dependent and yields EGFP fluorescence in response to exon 33.51 inclusion. A. Schematic of the Minigene design. B. Predicted splice products and effects on in-frame translation of fluorescent protein. C, D. Demonstration that EGFP fluorescence is ISE3 dependent both in the presence (IF3-5) and absence (IF6-8) of ISE1. E. Verification by RT-PCR that the fluorescence intensity of these minigenes correlates with the percentage exon 33.51 inclusion in DT3 cells. All results represent pooled stably transfected minigenes.



**Figure 5.** No activation of exon 33.51 splicing by any of the intron 8 elements occurs in AT3 cells as seen by EGFP fluorescence. Results from pools of stably transfected cells.



**Fig. 6.** Cell type specific activation of exon 33.51 inclusion can also be determined using mRFP based fluorescence. Results from pools of stably transfected cells.

## A Non-sequence-specific Double-stranded RNA Structural Element Regulates Splicing of Two Mutually Exclusive Exons of Fibroblast Growth Factor Receptor 2 (FGFR2)\*

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**Alternative splicing of fibroblast growth factor receptor 2 (FGFR2) mutually exclusive exons IIIb and IIIc represents a tightly regulated and functionally relevant example of post-transcriptional gene regulation. Rat prostate cancer DT3 and AT3 cell lines demonstrate exclusive selection of either exon IIIb or exon IIIc, respectively, and have been used to characterize regulatory FGFR2 RNA *cis*-elements that are required for splicing regulation. Two sequences termed ISE-2 and ISAR are located in the intron between exons IIIb and IIIc and are required for cell-type specific exon IIIb. Previous studies suggest that the function of these elements involves formation of an RNA stem structure, even though they are separated by more than 700 nucleotides. Using transfected minigenes, we performed a systematic analysis of the sequence and structural components of ISE-2 and ISAR that are required for their ability to regulate FGFR2 splicing. We found that the primary sequence of these elements can be replaced by completely unrelated sequences, provided that they are also predicted to form an RNA stem structure. Thus, a nonsequence-specific double stranded RNA stem constitutes a functional element required for FGFR2 splicing; suggesting that a double-stranded RNA binding protein is a component of the splicing regulatory machinery.**

Alternative splicing represents an important mechanism of modulating the expression of gene transcripts (1–5). It is estimated that at least 35% of human genes are alternatively spliced, although this is based on conservative estimates and it may be that alternative splicing is more the rule than the exception in the post-transcriptional processing of pre-mRNA transcripts (6, 7). Studies of the mechanisms employed by mammalian cells to differentially regulate splicing indicate that the selection of splice sites is influenced by proteins that bind to non-splice site RNA *cis*-elements and recruit or block spliceosome assembly. Such RNA *cis*-elements include exonic

and intronic splicing enhancers (ESEs<sup>1</sup> or ISEs) as well as exonic and intronic splicing silencers (ESSs or ISSs) that enhance or block splicing to neighboring splice sites. A number of ubiquitous RNA-binding proteins have been demonstrated to alter alternative splice site choice. Examples include the SR proteins (8–11), heterogeneous nuclear ribonucleoproteins (hnRNPs) (12–26), KSRP (KH-type splicing regulatory protein) (27), and TIA-1 (28, 29). While most proteins demonstrated to modulate splicing of mammalian transcripts are not differentially expressed, several mammalian tissue-specific RNA-binding proteins that function as splicing regulators have recently emerged (30–33). Alternatively spliced mammalian transcripts generally contain several *cis*-elements in introns and exons with positive or negative functions (enhancers and silencers) that modulate splicing. Such observations have led to models of combinatorial control, whereby regulation of alternatively spliced mammalian transcripts is achieved through the net influence of several proteins that bind to different *cis*-elements flanking regulated splice sites (1, 5). While some of the regulatory proteins implicated in splicing regulation are ubiquitously expressed, it appears that cell-type specific regulatory factors may often tip the balance in favor of specific splicing patterns in cells that express them.

A functionally relevant example of alternative splicing involves the mutually exclusive splicing of exons IIIb and IIIc of fibroblast growth factor receptor 2 (FGFR2). Inclusion of the 148-nt IIIb exon or the 145-nt IIIc exon in the region encoding the second half of the third extracellular ligand binding domain results in either FGFR2-IIIb or FGFR2-IIIc, respectively (Fig. 1). For a given cell type the splicing pattern is exclusive and only one of the isoforms is expressed (34, 35). Although both receptors bind FGF-1 with equivalent affinity, they otherwise display distinct and exclusive differences in binding to most other FGFs thus far characterized (36). Appropriate tissue-specific expression of FGFR2-IIIb or FGFR2-IIIc is crucial for maintenance of normal tissue homeostasis and dysregulated splicing of this transcript has been proposed to be one event that can lead to progression of cancers of epithelial origin, including prostate and bladder cancers (34, 37–39). Several studies have utilized rat and human FGFR2 minigenes to identify *cis*-elements in the introns and exons of these transcripts that influence splicing of exons IIIb and IIIc and are shown schematically in Fig. 1 (26, 40–44). Within the intron separating exons IIIb and IIIc, the ISAR (intronic splicing

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY161008 and AY161009.

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<sup>1</sup> The abbreviations used are: ESE, exonic splicing enhancer; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; ESS, exonic splicing silencer; ISE, intronic splicing enhancer; ISS, intronic splicing silencer; ISAR, intronic splicing activator and repressor; hnRNP, heterogeneous nuclear ribonucleoprotein; PTB, polypyrimidine tract-binding protein; UTR, untranslated region; nt, nucleotide.

activator and repressor) element in rat transcripts was shown to play a dual role in splicing regulation; activation of the upstream exon IIIb and repression of the downstream exon IIIc in cells that express FGFR2-IIIb (40). A nearly identical sequence in human transcripts termed IAS3 displays the same splicing regulatory activities (43, 45). The function of IAS3 was proposed to involve formation of an RNA secondary structure with another element upstream in the same intron termed IAS2 (43). A rat sequence that is identical to IAS2 is likewise predicted to form a secondary structure with ISAR and evidence has been put forth that suggests this secondary structure also is involved in splicing regulation of rat transcripts (46). Although this rat element has previously been referred to as rIAS-2, we will henceforth refer to the element as ISE-2 to conform with a convention using "ISE" to refer to intronic sequences that positively regulate splicing. The ability of ISE-2 and/or ISAR to regulate FGFR2 splicing has thus far only been demonstrated in cells that express FGFR2-IIIb. It is therefore of great interest to identify proteins that interact with these elements as they may include proteins that are exclusively expressed in cell types that splice exon IIIb. Given data that suggest that these elements function through formation of an RNA secondary structure, it is important to perform a systematic analysis of the precise sequence and structural requirements of these elements that are required for their ability to regulate splicing.

In this study, we make use of cell lines, DT3 and AT3, that have previously been used to study FGFR2 splicing regulation (26, 40, 46, 47). DT3 cells exclusively express FGFR2-IIIb and AT3 cells express FGFR2-IIIc and transfected minigenes that recapitulate the splicing pattern of the endogenous FGFR2 gene transcript have been described (40). We performed a comparative sequence analysis of elements that are related to ISE-2 and ISAR. We found that these intronic sequences are highly conserved among vertebrates. Furthermore, we found related sequences in the corresponding intron of fibroblast growth factor receptor 1 (FGFR1). The predicted secondary structures by these related sequences form 17–21-nt base-paired stems containing short internal loops or bulges. In the case of rat FGFR2 ISE-2 and ISAR elements, we found that deletion of sequences that form a bulge in a predicted 18-nt stem does not impair the function of these elements in FGFR2 splicing regulation. We then determined the effect of replacing each element with unrelated sequences that would also form a complementary stem. Surprisingly, we found that these unrelated sequences were equally capable of mediating cell-type-specific splicing regulation of exons IIIb and IIIc. Thus, we conclude that the sequences of ISE-2 and ISAR themselves are not required for the function of these elements. Rather, the function of these elements can be recapitulated by any RNA sequences that form related RNA secondary structures. Thus, a non-sequence-specific RNA secondary structure constitutes the functional element that mediates splicing regulation by ISE-2 and ISAR.

#### EXPERIMENTAL PROCEDURES

**Plasmid Construction**—All plasmids and minigene constructs employed standard cloning techniques as previously described (40). The pI-11-FS and pI-11-NC-W/W37 (the latter previously denoted as pI-11-FS-NC-SAR-20) splicing constructs were obtained as previously described (40). pI-11-pPANC-W/W37 was created as a modification of pI-11-NC-W/W37 by inserting a *PacI* restriction site directly 5' to the identified ISE-2 region (5'-ACACCCGTAAAAAGGTACAA-3') and *AscI* and *BspEI* sites were inserted immediately 3' of this ISE-2 region. By convention all minigenes derived from pI-11-PANC are described with a suffix in which sequences in the position of ISE-2 precede sequences in the position of ISAR, separated by a hash mark. Sequences inserted in these positions are as summarized in Fig. 3A. Thus, minigenes in which ISAR is modified (pI-11-PANC: W/M2, W/M3, W/B37, W/G19,

W/B19, W/W19, and W/WS) were achieved by deleting the 37-nucleotide sequence containing ISAR (W37 or SAR-20) from pI-11-pPANC-W/W37 with *NotI* and *ClaI* and inserting annealed oligonucleotides with complementary *NotI* and *ClaI* sites, as represented by the following oligonucleotide pairs: M2: Mut2-F, 5'-GGCCGCGCAAGAGAACGGAC-TCTGTGGGCTGAAAGATCCATGTAT-3' and Mut2-R, 3'-CGATACATGGATCTTTTCAGCCACAGAGTCCGTTCTCTTTGGC-3'; M3: Mut3-F, 5'-GGCCGCGCAAGAGAACGGACTCTGTGGGCTGATTTTTCACGCT-AT-3' and Mut3-R, 5'-CGATAGCGTGAAAATACAGCCACAGAGTC-CGTCTCTTTTGGC-3'; B37: Blu-F, 5'-GGCCGCAACTGGTGGCCCTA-CTACGGCTACACTAGAAGGACACAT-3' and Blu-R, 5'-CGATGTGT-CCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTGC-3'; G19: Globin-F, 5'-GGCCGCGACCTATGTCTCCACATGCCAT-3' and Globin-R, 5'-CGATGGCATGTGGAGACATAGGTGC-3'; B19: Blue19-F, 5'-GGCCGCGCGTATCGTAGTTATCTACAT-3' and Blue19-R, 5'-CGA-TGTAGATAACTACGATACGGGC-3'; W19: SAR-38-F, 5'-GGCCGCTG-TGGGCGATTTTTCATGTAT-3' and SAR-38-R, 5'-CGATACAGTGA-ATGCCCCACAGC-3'; WS-F, 5'-GGCCGCGCATGAAAAATGCCAC-AAAT-3' and W-R, 5'-CGATGTGTGGGCATTTTTCATGGGC-3'. The ISE-2 mutant constructs (pI-11-pPANC: M2'/W37, M3'/W37, B37'/W37, G19'/W37, B19'/W37, and W37S/W37) were created by deleting ISE-2 from pI-11-pPANC-W/W37 with *PacI* and *AscI* and inserting annealed oligonucleotides with complementary *PacI* and *AscI* sites, as represented by the following oligonucleotide pairs: M2': M2'-F, 5'-TA-ACCATGGATCTTTGCCCACAAGG-3' and M2'-R, 5'-CGCGCCTTGT-TGGCAAAGATCCATGGTTAAT-3'; M3': M3'-F, 5'-TAACGCGTGA-ATGCCCCACAAGG-3' and M3'-R, 5'-CGCGCCTTGTGGGCATTTTTC-ACGCGTTAAT-3'; B37': IS-BLU-F, 5'-TAAGTGTCTTCTAGTGTAG-CGTAGTTAGGCCACCACTTGG-3' and IS-BLU-R, 5'-CGCGCCAAG-TGGTGGCCCTAACTACGGCTACACTAGAAGGACACATTAAT-3'; G19': Globin-F, 5'-TAAGGCATGTGGAGACATAGGTGGG-3' and Globin-R, 5'-CGCGCCCACTATGTCTCCACATGCCCTAAT-3'; B19': BLU19'-F, 5'-TAATGTAGATAACTACGATACGGGC-3' and BLU19'-R, 5'-CGCG-CCCCGTATCGTAGTTATCTACATTAAT-3'; W37S: W37'-F, 5'-TAAC-TGTGGGCTGATTTTTCATGTGG-3' and W37'-R, 5'-CGCGCCACAT-GGAAAAATCAGCCACAGTTAAT-3'. Plasmids pI-11-pPANC: M2'/M2, M3'/M3, B37'/B37, G19'/G19, B19'/B19, and W37S/WS were generated by deleting wild type ISE-2 (W) from pI-11-pPANC: W/M3, W/M4, W/B37, W/G19, W/B19, and W/WS with *PacI* and *AscI* and inserting, respectively, annealed oligonucleotides corresponding to M2', M3', B37', G19', B19', and W37S as described above. Minigene pI-11-PANC-del-AN (13) was generated by digesting pI-11-PANC-W/W37 with *AscI* and *NotI* to delete sequences between these sites in intron 8, followed by gel purification, blunting of the digested ends with *Pfu* polymerase (Stratagene), and religation using methods previously described (40). Minigene pI-11-PANC-del-AN (17) was generated by digesting pI-11-PANC-W/W37 with *AscI* and *NotI* to delete sequences between these sites in intron 8 and replacing with the following annealed oligos: FGFR2-AN-F, 5'-CGCGCCGC-3' and FGFR2-AN-R, 5'-GGCCGCGG-3'. All plasmid minigene constructs were prepared with Qiagen plasmid maxi kits. Sequences of all the minigenes were confirmed by sequence analysis using the University of Pennsylvania sequencing facility.

**Genomic Sequence Data Acquisition**—Complete human and mouse genomic sequence data corresponding to the intron between exons IIIb and IIIc of FGFR1 and FGFR2 was obtained from public sequencing projects through the National Center for Biotechnology Information. The same sequences from rat and frog (*Xenopus laevis*) were obtained using long distance PCR with primers corresponding to exons IIIb and IIIc from each species, cloning into PCR-Blunt-Zero (Invitrogen), and sequencing. Identification of conserved intronic sequences was carried out using multiple alignment comparisons (Megalign; DNASTAR) and by visual inspection.

**Cell Culture and Transfection**—AT3 and DT3 cells were maintained in Dulbecco's Modified Eagle Medium (Invitrogen) supplemented with 10% fetal bovine serum (LTI). Transfections were carried out using LipofectAMINE 2000 (Invitrogen) according to the supplier's recommendations. Selection was performed using Geneticin (Invitrogen) at an active concentration of 400–500  $\mu$ g/ml until isolated colonies were obtained, and no cells remained from a control transfection with a plasmid lacking neomycin resistance. Pooled colonies were then harvested for RNA preparation.

**RNA Purification and RT-PCR Analysis**—Total cellular RNA and RT-PCR was performed as previously described with several exceptions described below (40). Primers PI-11-(H3)-F (5'-GCTGGAATTCGAGCTCACTCTCTTC-3') and PIP11-R (5'-CCCGGGACTAGTAAGCTTAG-GCTCTTGGCGTT-3') were used for analysis of the transfected minigenes and were complementary to sequences in the upstream and



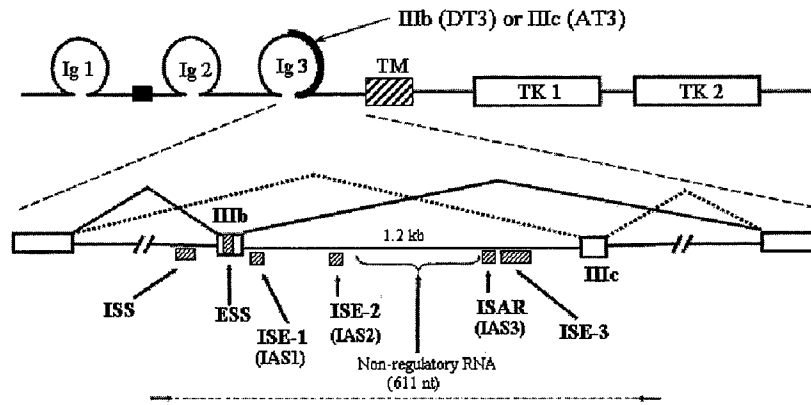


FIG. 1. Schematic representation of alternative splicing variants of FGFR2. At the top is a protein domain map with the region encoded by mutually exclusive exons IIIb or IIIc indicated by a thick line. A dark box indicates an acidic domain and the hatched box indicates the transmembrane domain (TM). Ig, immunoglobulin-like domain. TK, tyrosine kinase domain. At the bottom is a map of the pre-mRNA in the region containing exons IIIb and IIIc. Hatched boxes represent intronic splicing elements shown to influence splicing of exon IIIb or IIIc. ISE, intronic splicing enhancer. ISS, intronic splicing silencer. ESS, exonic splicing silencer. The human elements analogous to the rat ISE-1, ISE-2, and ISAR elements are shown in parentheses. The horizontal arrows and dashed line underneath the pre-mRNA represent the FGFR2 genomic sequence that was amplified by PCR and used to generate minigene pI-11-FS.

downstream adenoviral exons contained in all minigenes. PCR amplifications were performed using conditions previously described but with cycles consisting of initial denaturation at 94 °C for 5 min, followed by 25–30 cycles of denaturation at 94 °C for 30 s, annealing at 55° for 30 s, and extension at 72 °C for 1 min. After completion of the final cycle, there was a final extension at 72 °C for 7 min. In all PCR reactions, a water and –RT control were included and were negative in all presented data. The PCR products were digested with either *Ava*I or *Hinc*II (New England Biolabs) restriction endonucleases. Aliquots representing equal amounts of each digested or undigested PCR reaction mixtures were directly loaded on non-denaturing 5% polyacrylamide gels and electrophoresed at 110 V for 3–4 h, followed by drying and autoradiography (Amersham Biosciences Hyperfilm-MP). Quantification of data was performed with a Molecular Dynamics PhosphorImager. Because equal amounts of *Ava*I- and *Hinc*II-digested PCR products were loaded onto each gel, quantification of cDNAs containing exon IIIb (UBD or UCD, respectively, where U and D are the 5' and 3' exons of pI-11) was obtained by using the quantification of the band at 286 bp, which remained following *Hinc*II digestion as the numerator. The denominator consisted of the sum of both bands (286 and 283) that remained following *Ava*I and *Hinc*II digestion (UBD + UCD). When these results were also expressed with the contribution of products with IIIb and IIIc skipped, the average value of the 138-bp band was also used in the sum of the denominator (UBD + UCD + UD), corrected for molar equivalents.

## RESULTS

**Regulatory FGFR2 cis-Elements Predicted to Form a Secondary Structure Are Highly Conserved at a Sequence and Structural Level**—Previous studies of rat and human FGFR2 splicing regulation have implicated several RNA cis-elements that are required for appropriate cell type-specific splicing of exons IIIb and IIIc (Fig. 1). Within the intron between these two alternative exons, intron 8, the previously described ISAR in rat transcripts or IAS3 in human transcripts, is required for exon IIIb inclusion in cells that express FGFR2-IIIb (40, 43). We currently use ISAR to refer to a 20-nucleotide element located from 945 to 964-nt downstream of exon IIIb, which corresponds to the approximate location of IAS3 in human FGFR2. The human IAS2 is located over 700-nt upstream of IAS3 (43). The rat ISE-2 sequence is located in the same approximate location as IAS2 in intron 8 (191–208-nt downstream of exon IIIb) (40, 46). The predicted secondary structure formed by ISE-2 and ISAR as well as the related structure from the homologous human elements is shown in Fig. 2B. It is noteworthy that the ISE-2 and ISAR are separated by 736 nt suggesting that a loop of this size separates a putative stem formed between these elements (775 nt in human transcripts). ISE-1 and ISE-3 are also required for efficient exon IIIb splic-

ing in DT3 cells. In order to further investigate the hypothesis that ISE-2 and ISAR function through formation of an RNA stem structure, we also obtained sequence from the same FGFR2 intron from mouse (*Mus musculus*) and frog (*X. laevis*) and identified highly similar sequences that correspond to these two elements. These sequences are aligned in Fig. 2A and it can be seen that these intronic elements are highly conserved at the sequence level. For each organism the ability of the ISE-2 and ISAR element to form related secondary structures was determined using the Mfold version 3.1 program (48). As shown in Fig. 2B, the human, mouse, and rat sequences predict a highly similar stem structure with slightly variant internal loops or bulges. The frog sequences displayed the greatest difference with the rat sequences of the ISE-2 and ISAR elements, yet when secondary structure was determined, they nonetheless also formed a similar secondary structure that, like the other sequences, consisted of two interrupted stems. Of note, the most highly similar structures were those of rat and mouse, which consisted of 18 Watson-Crick base-paired bases with a 2-nucleotide bulge. The only two bases that differed between rat and mouse ISE-2 or ISAR were the two nucleotides that comprised the non-base-paired bulge in each element. This observation, together with the more divergent internal loops contained within the human and frog secondary structures suggested that sequences and/or structures of these non-base-paired residues are not critical for their function.

**Sequences Homologous to ISE-2 and ISAR Are Present between Alternatively Spliced Exons IIIb and IIIc of FGFR1**—Alternative splicing of exons that represent homologues of exons IIIb and IIIc has been observed for FGFR1, FGFR2, and FGFR3. The two members of the group with the highest level of sequence and genomic structural similarity are FGFR1 and FGFR2 (49). In addition, the exon and intron sizes in the genomic region that encodes exons IIIb, IIIc, and the constitutive upstream and downstream exons are highly similar. Alternative splicing of FGF-R1 IIIb and IIIc exons is highly regulated and cell types with exclusive expression of FGF-R1 (IIIb) have been described (50). FGFR1 and FGFR2 most likely arose from a gene duplication event of a single common ancestral gene that followed a previous exon duplication that gave rise to alternative exons IIIb and IIIc. We thus speculated that sequence elements that direct splicing regulation of FGFR1 exons IIIb and IIIc may be conserved between these gene paralogues. Sequence analysis of the intron between FGFR1 exons IIIb and IIIc from human, mouse, rat, and frog DNA revealed the pres-

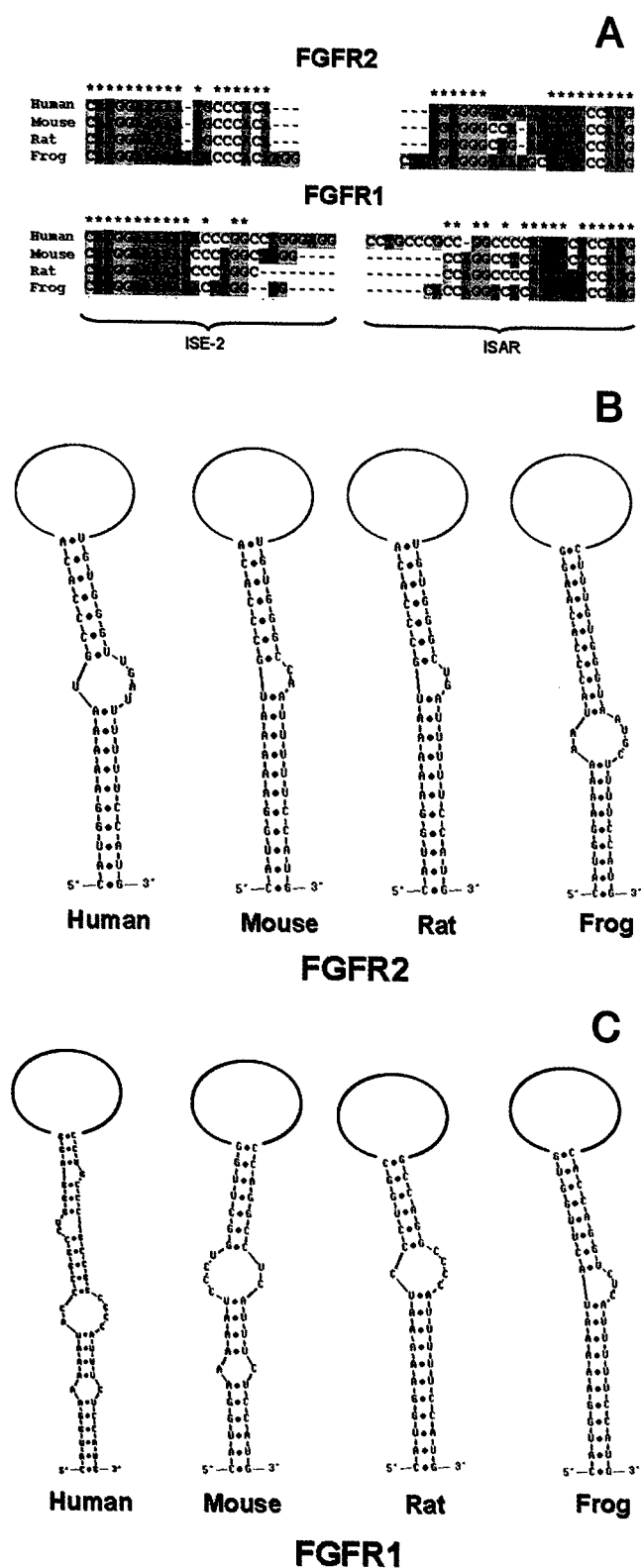
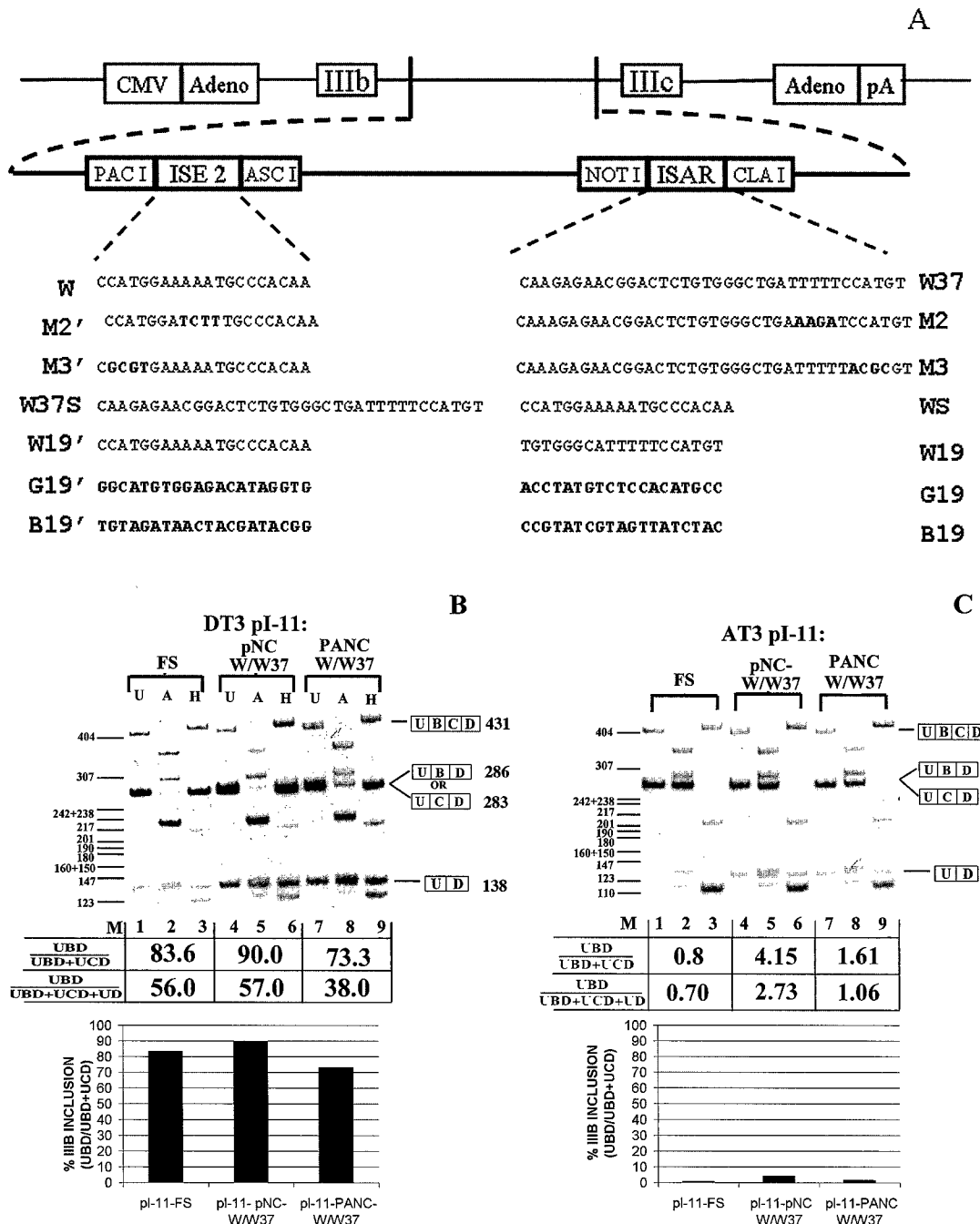


FIG. 2. Sequences similar to ISE-2 and ISAR are highly conserved and predict similar stem structures in FGFR1 and FGFR2 transcripts. **A**, alignment of FGFR1 and FGFR2 sequences from the intron between exon IIIb and IIIc from human, mouse, rat, and frog that are highly similar to ISE-2 and ISAR. Sequences designated as ISE-2 are shown at left and those designated as ISAR are shown at right. Stars at the top of each alignment represent nucleotides that are identical in all four species. **B**, secondary structures predicted to form between ISE-2 and ISAR from FGFR2 transcripts. **C**, secondary structures predicted to form between ISE-2 and ISAR from FGFR1 transcripts. The loops drawn at the top of each stem structure are not at scale as they represent 500 or more nucleotides between the elements in each transcript.

ence of sequence elements highly similar to ISE-2 and ISAR in the same approximate locations within the intron (Fig. 2A). Thus, for example, the rat intron separating exons IIIb and IIIc of FGFR1 is 1004 nt (compared with 1200 for FGFR2) and homologues to ISE-2 and ISAR are positioned between 126–143 and 696–717 downstream of exon IIIb, respectively. We used Mfold to determine whether these elements could also form similar secondary structures. As shown in Fig. 2C, secondary structures that were similar to those formed with the FGFR2 elements were predicted. Thus, for example, the rat element contained a base-paired region at the base of the stem that was identical to that of FGFR2, whereas sequences that were not identical to the FGFR2 ISE-2 and ISAR elements nonetheless predicted a different base-paired region with an intervening small internal loop. In the case of the human structure, two additional small bulges were predicted in the top of the secondary structure that would be predicted to disrupt stem formation in this region, yet the extent of base pairing could be further extended to yield a total of 21 base-paired residues compared with 19 for the frog structure and 17 for the mouse and rat structures. Thus, in general, these phylogenetic comparisons were consistent with a hypothesis that ISE-2 and ISAR elements regulate splicing through formation of a secondary structure that arises through base pairing interactions between these elements. Furthermore, these results suggest that the sequences and/or structures that comprise these elements have been maintained through a long evolutionary history.

*Minigene pI-11-PANC-W/W37 Recapitulates the Splicing Pattern of the Endogenous Gene in AT3 and DT3 Cells*—In order to further investigate the sequences and/or structures of ISE-2 and ISAR that are required for splicing regulation we further modified a minigene, pI-11-FS, that was previously shown to recapitulate the splicing pattern of endogenous FGFR2 when stably transfected into AT3 and DT3 cells (40). These minigenes contain a 1804 nt region of FGFR2 that includes exons IIIb and IIIc as well as flanking intron sequences upstream of exon IIIb, downstream of exon IIIc, and the entire intron (intron 8) that separates the exons. This genomic region was positioned in the intron of an adenoviral expression cassette that consists of two constitutively spliced adenoviral exons (Fig. 3A). Pools of stably transfected AT3 and DT3 cells were used to harvest RNA and used with a previously validated RT-PCR assay that accurately distinguishes between spliced RNAs that contain exon IIIb or exon IIIc. Using minigene-specific PCR primers a predominant product of 286 or 283 bp is observed when spliced products contain exon IIIb or exon IIIc, respectively. In order to distinguish whether these products contain exon IIIb or IIIc, they are digested with *AvaI* and *HincII*, which specifically digest only those products that contain exon IIIb or IIIc, respectively. As shown previously, transfection of pI-11-FS into DT3 and AT3 cells yields products that nearly exclusively contain exon IIIb or IIIc, respectively (Fig. 3B, lanes 1–3, and C, lanes 1–3). Of note, two additional PCR products are seen in these transfections. One such product contains both exon IIIb and IIIc (431 bp) and another consists of products that represent mRNAs in which the adenoviral exons have been directly ligated and both exon IIIb and IIIc skipped (138 bp). As will be described, one consequence of loss of splicing regulation in DT3 cells is a switch to exon IIIc inclusion, but also an increase in products in which both exon IIIb and IIIc are skipped. Therefore, using phosphorimager analysis we quantify the percentage of exon IIIb containing products relative to that of exon IIIc, but also relative to that of exon IIIc together with skipped products.

In order to further characterize the ISE-2 and ISAR regula-



**FIG. 3. Splicing regulation is maintained following insertion of restriction sites immediately flanking ISE-2 and ISAR in rat FGFR2 minigenes.** A, schematic demonstrating the construction of pI-11-PANC-W/W37. At top is shown minigene pI-11-FS. Boxes represent exons and lines indicate introns. CMV, cytomegalovirus promoter; pA, polyadenylation signal. Exons IIIb and IIIc as well as the adenoviral exons (Adeno) are indicated. In the middle is a representation of FGFR2 intron 8 showing the ISE-2 and ISAR elements after the indicated restriction sites have been introduced flanking each element. (Note, boxes in this region indicated the intron modifications, not exons.) At bottom the sequences positioned in the respective locations of derived minigenes are shown. Sequences shown in **bold** represent mutations of either element or unrelated sequences used to replace ISE-2 or ISAR. B, minigene pI-11-PANC-W/W37 maintains exon IIIb splicing when stably transfected into DT3 cells, but with slightly reduced efficiency compared with pI-11-FS and pI-11-NC-W/W37. Results for each minigene represent RT-PCR products that are undigested (U), digested with *Ava*I (A), or digested with *Hinc*II (H) as shown above each lane in this figure. All subsequent results use the same order of undigested, *Ava*I-digested, and *Hinc*II-digested products. M, pBR/MspI molecular weight markers. At right spliced products represented by each band are shown. U and D indicate upstream and downstream adenoviral exons, respectively. Quantified results are shown below each set of results using percentages of exon IIIb inclusion as described under "Experimental Procedures." C, exon IIIc, and not exon IIIb, is included in spliced products from these minigenes in AT3 cells.

tory sequences of the ISE-2 element we created a new minigene pI-11-PANC-W/W37. We had previously generated a minigene, pI-11-FS-NC: SAR-20 in which a 57-nt region from intron 8 that contained ISAR was deleted and replaced with a shorter 37-nucleotide region (W37) that contained all functional ISAR sequences, but flanked by *Not*I and *Cla*I restriction sites at the

5'- and 3'-end of the element. This minigene, designated here as pNC-11-W/W37, also recapitulated cell-specific splicing of exons IIIb and IIIc (Fig. 3B, lanes 4–6 and C, lanes 4–6). The minigene pPANC-W/W37 was generated by further modifying pNC-W/W37 by engineering *Pac*I and *Asc*I restriction sites immediately 5' and 3' of a 20-nt sequence containing ISE-2.

Therefore, this minigene contains both ISE-2 and ISAR, but with restriction sites flanking each element. Because the wild-type ISE-2 and ISAR elements are present in this minigene we use a convention W/W37 in which W refers to wild type sequences and the hash mark separates the sequences contained, in order, in the position of ISE-2 and ISAR. This minigene and other derived minigenes are displayed schematically in Fig. 3A. When pI-11-PANC-W/W37 was transfected into AT3 cells we again observed nearly exclusive use of exon IIIc (Fig. 3C, lanes 7–9). However, in DT3 cells we noted that a slightly increased proportion of products contained exon IIIc and also an increase in skipped products when compared with pI-11-FS or pI-11-NC-W/W37 (Fig. 3B, lanes 7–9). Thus, it is evident that introduction of either the *PacI* or *AscI* site slightly impairs the function of ISE-2 and ISAR to mediate exon IIIb inclusion in DT3 cells. Nonetheless, this construct still clearly recapitulates cell specific regulation and allows ISE-2 and ISAR to be directly manipulated to further investigate the role of these sequences on splicing regulation. This new construct was thus subsequently used as a positive control for further investigation of the role ISE-2 and ISAR play in splicing regulation. Because all minigenes to be described in subsequent sections all yielded exclusively exon IIIc containing spliced products in transfected AT3 cells, we will henceforth only show results from transfections in DT3 cells.

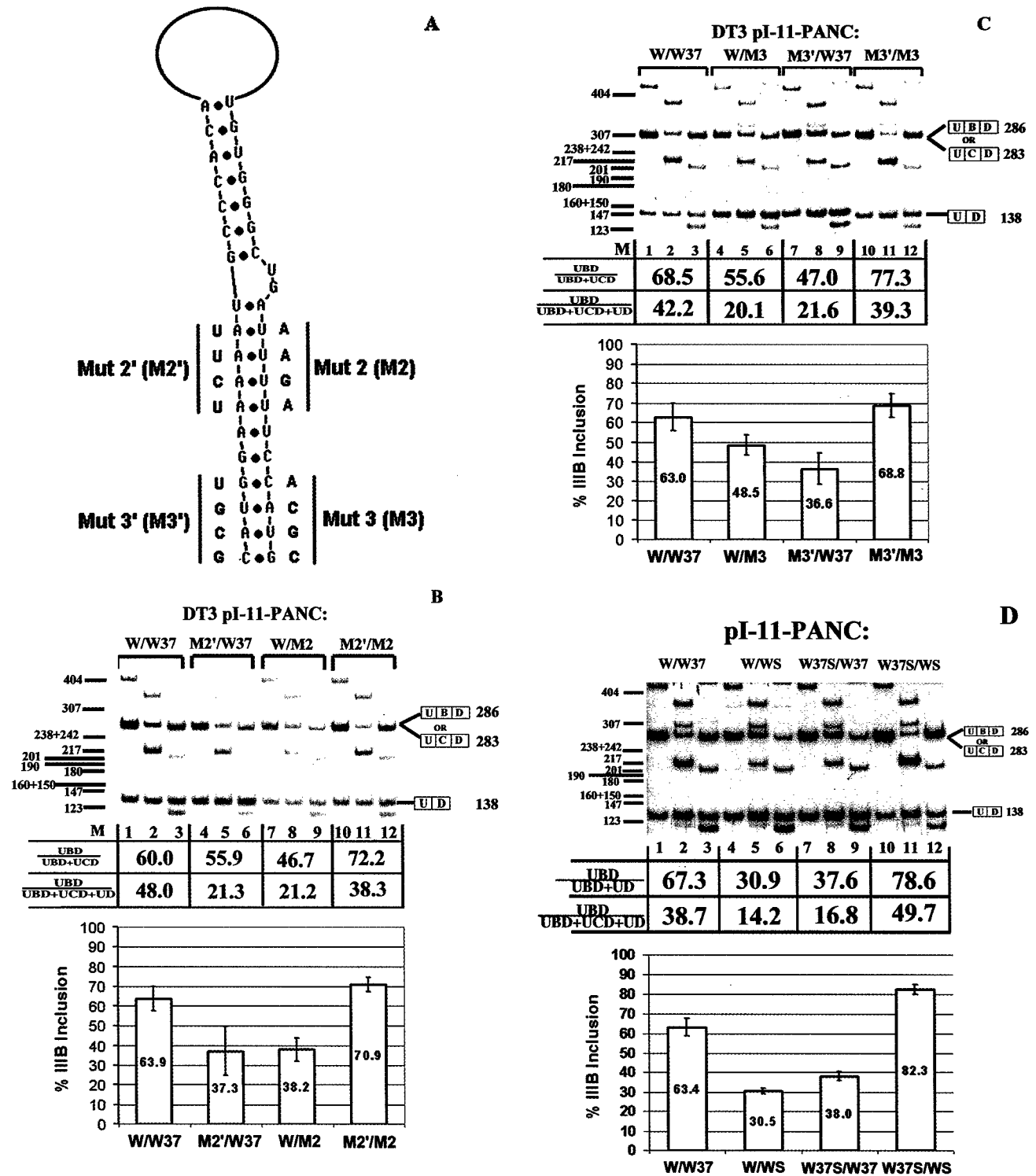
**Mutations in ISAR Predicted to Disrupt a Proposed Secondary Structure Cause Loss of Splicing Regulation and Complementary Mutations in ISE-2 Restore Splicing Regulation**—We sought to further test whether formation of a secondary structure between ISE-2 and ISAR is involved in regulation of rat FGFR2 splicing as previously proposed for the related *cis*-elements in human FGFR2 transcripts (43). We first determined whether mutations in ISAR that result in loss of splicing regulation in DT3 cells can be “rescued” by making corresponding complementary mutations in ISE-2. A similar approach using human minigenes showed that complementary mutations increased exon IIIb splicing, although different pairs of complementary mutations predicted to restore base pairing between these elements did not all restore exon IIIb inclusion to the levels obtained with the wild-type sequences (43). Two previously described mutations in ISAR, Mut 2 and Mut 3, were previously shown to result in a significant loss of exon IIIb inclusion (40). These mutations are shown next to the wild type ISAR sequence in the context of the putative secondary structure in Fig. 4A and it can be seen that either mutation would result in disruption of a stem formed at the base of this structure. These mutated sequences (denoted M2 and M3) were placed in pI-11-PANC in place of wild-type ISAR (W37), and complementary mutations (M2' and M3') were used to replace the wild-type ISE-2 (W). Introducing any of these ISE-2 or ISAR mutations led to a reduction in exon IIIb inclusion in stably transfected DT3 cells when compared with pI-11-PANC-W/W37 (Fig. 4, B and C, lanes 4–9). In Fig. 4 and subsequent figures, a representative experiment is shown at the top and the average percentage of exon IIIb inclusion from four independently performed transfections is shown graphically at the bottom. It should be noted that some reductions in exon IIIb splicing in these experiments appear small, but it is worth noting that in addition to a proportional increase in products that include exon IIIc, the proportion of skipped products (containing neither exon IIIb nor exon IIIc) is greatly increased by these mutations. However, when the ISAR mutations (M2 and M3) were tested together with complementary ISE-2 mutations (M2' and M3') exon IIIb splicing was restored (Fig. 4, B and C, lanes 10–12).

We also hypothesized that if formation of a secondary struc-

ture determines the ability of ISE-2 and ISAR to function in splicing regulation, the positions of these elements should be able to be switched without impairing the ability of the elements to function together to regulate exon IIIb inclusion. We therefore generated constructs in which ISAR (W37S) was used to replace ISE-2 and *vice versa* to generate minigene construct pI-PANC-W37S/WS. As controls, either element was present in both positions (pI-11-PANC-W/WS and pI-11-PANC-W37S/W37; note the designation “S” indicates the same sequence, but whose position has been switched in the minigenes). As shown in Fig. 4D, switching the positions of these elements indeed preserved splicing regulation whereas duplication of either element was associated with loss of splicing regulation. In fact, the level of exon IIIb inclusion when these positions were reversed was higher than that of pI-11-PANC W/W37.

**ISAR Sequences Predicted to Base Pair with ISE-2 Are Sufficient to Mediate Function of This *cis*-Element**—As shown in Fig. 2B, the putative secondary structure between rat ISAR and ISE-2 consists of an 18-nt base-paired stem with an internal 2-base bulge. The 37-nt ISAR sequence previously described (W37) encodes additional nucleotides that normally flank the sequences that are contained within the secondary structure. We replaced W37 with W19 in which the two bases that should form a bulge have been deleted as have all nonbase-paired ISAR sequences except a 3'-terminal U. Thus an uninterrupted 18-nucleotide stem is predicted to result if a secondary structure forms from ISE-2(W) and W19. The predicted secondary structure that would result from replacing W37 with W19 is shown in Fig. 5A. Transfection of the resulting minigene, pI-PANC-W/W19, in DT3 cells indicates that deletion of nonbase-paired sequences, including those encoding a putative bulge in the stem, does not impair splicing regulation and in fact exon IIIb inclusion was reproducibly higher (Fig. 5B, lanes 1–3, compare with lanes 1–3 from Fig. 4, B, C, or D).

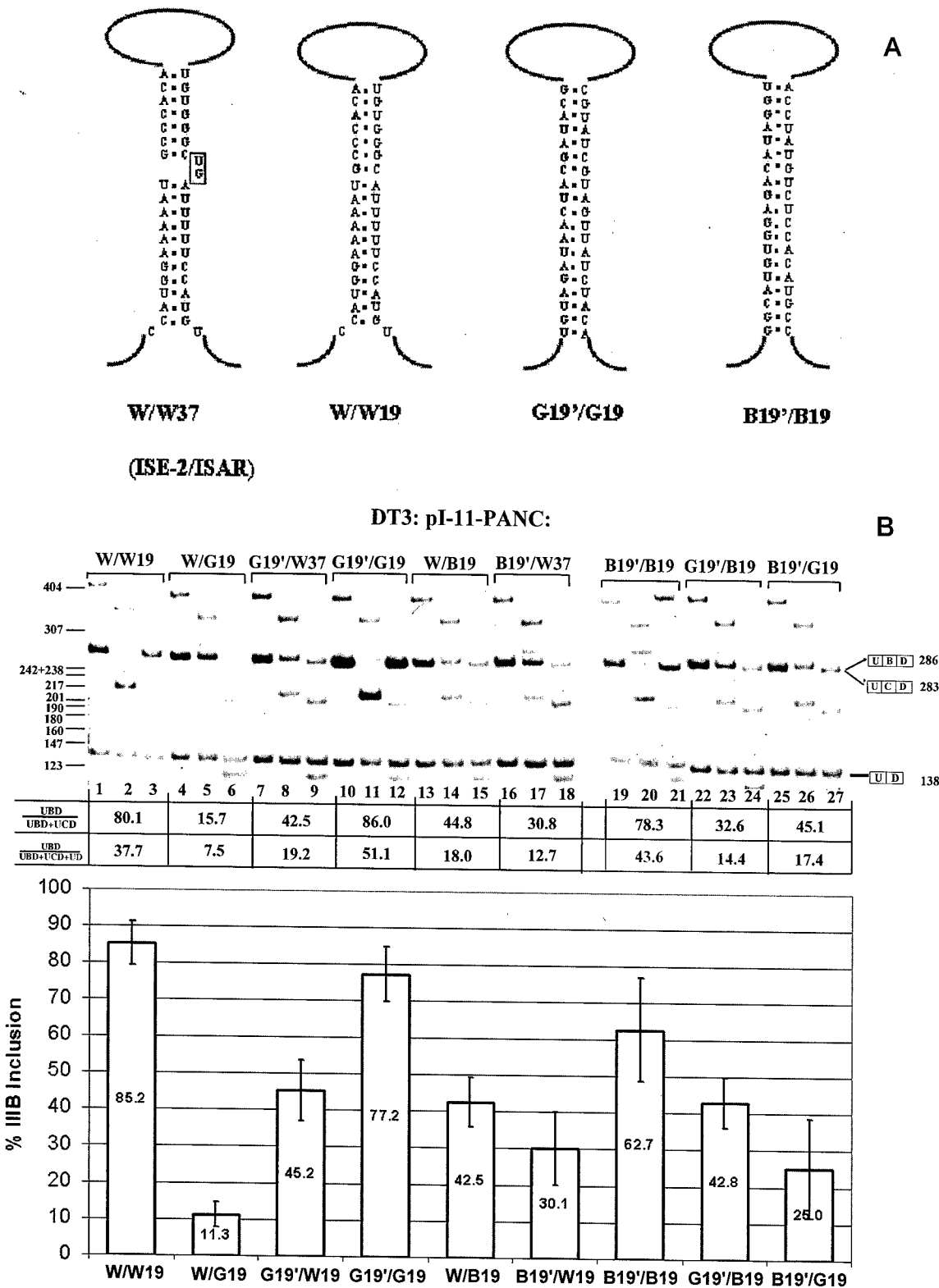
**The Splicing Regulatory Functions of ISE-2 and ISAR Can Be Recapitulated When They Are Replaced by Randomly Selected Sequences That Also Form a Stem Structure**—The predicted FGFR2 stems derived from ISE-2 and ISAR shown in Fig. 2B consist of interrupted stems that contain between 17 and 20 base-paired nucleotides. The base-paired nucleotides most distal to the loop (the bottom of the structures as shown) display the highest degree of sequence conservation both between these species as well as with putative FGFR1 elements. Sequences located toward the top of the structures display less sequence conservation, but in general maintain the potential to form base pairing interactions that maintain stems of similar overall length. Given the high degree of sequence conservation of portions of each element we suspected that while an 18–20-nucleotide stem structure may indeed be required for function of ISE-2 and ISAR, it was still possible that specific sequences within this stem may nevertheless be required in order to regulate splicing. In order to determine whether any specific sequences of ISE-2 and ISAR are required for the function of this element we selected two unrelated 19 nucleotide sequences, one from the second intron of human  $\beta$ -globin (G19) and the other (B19) from the pBluescript (Stratagene) plasmid sequence. These sequences were used to replace ISAR in pI-11-PANC-W/W37. We then replaced ISE-2 in the resulting plasmids with sequences, G19' and B19', that are predicted to form a perfect 19-nt stem with the G19 or B19 sequences, respectively. These sequences and the predicted RNA stem structures are shown in Fig. 5A. As controls, each of these sequences was also cloned into pI-11-FS-PANC in all combinations that would not form a 19 nt stem (e.g. G19 replacing ISAR and B19' replacing ISE-2). The resulting plasmids were stably transfected into DT3 cells and exon IIIb inclusion determined by



**FIG. 4. Complementary mutations in ISE-2 or ISAR that restore a predicted RNA stem structure maintain exon IIIb inclusion in DT3 cells.** *A*, schematic of the predicted secondary structure between rat FGFR2 ISE-2 and ISAR with the indicated mutations positioned adjacent to the sequences they replace. *B*, mutations M2 or M2' result in loss of splicing regulation, but when both mutations are present together, splicing regulation is restored. *C*, mutations M3 or M3' result in loss of splicing regulation, but when both mutations are present together, splicing regulation is restored. *D*, switching the positions of ISE-2 and ISAR maintains splicing regulation. WS indicates wild-type ISE-2 in the position normally occupied by ISAR. W37S indicated wild-type ISAR in the position normally occupied by ISE-2. Abbreviations and lane designations are as described in the legend to Fig. 3. In this and subsequent figures, data from a representative experiment is presented at the top. Bar graphs represent the results from four independently performed sets of transfections. Numbers in the bars represent the mean % IIIb inclusion from all experiments, and error bars indicate S.D.

RT-PCR. The results were then compared with the plasmid in which a bulgeless stem comprised of ISE-2 and ISAR sequences was predicted to form (pI-11-PANC-W/W19). Surprisingly, both random sequences (G19 or B19), when present together with

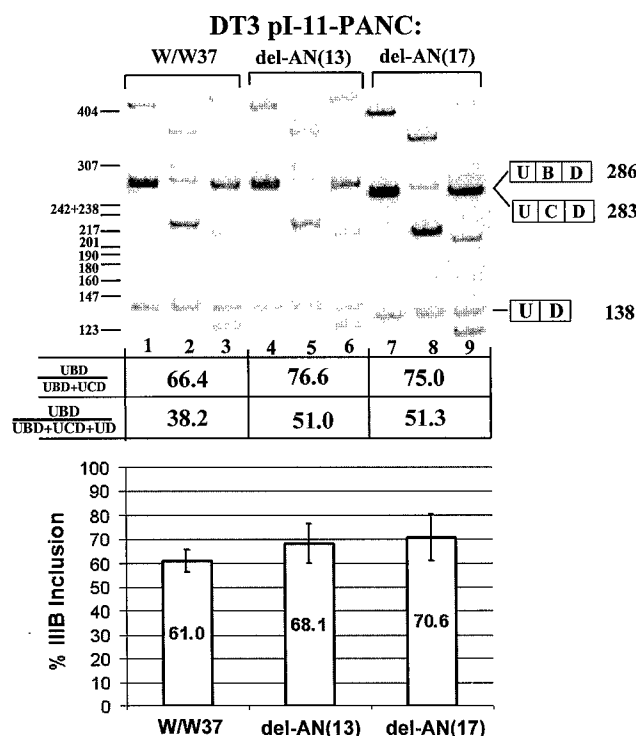
their complementary sequence (G19' or B19') were able to restore exon IIIb splicing in place of the ISE-2 and ISAR sequences (Fig. 5B, lanes 10–12 and 19–21). Replacing any of these sequences in combinations that did not predict a 19-nt



**FIG. 5. An 18–19-nucleotide base-paired stem is sufficient to mediate splicing regulation in DT3 cells.** A, schematic presentation of secondary structures predicted to form in pI-11-PANC-W/W37, pI-11-PANC-W/W19, pI-11-PANC-G19'/G19, and pI-11-PANC-B19'/B19. The bulge containing the UG dinucleotide is boxed. B, splicing regulation is maintained when the bulged nucleotides are removed and when ISE-2 and ISAR are replaced by unrelated sequences that are predicted to form a perfect 19-nucleotide stem structure. Abbreviations and lane designations are as described in the legend to Fig. 3.

stem gave results that were equivalent to that seen in which either ISE-2 or ISAR was deleted. Therefore, it is evident that the functions of ISE-2 and ISAR can be accounted for solely by their ability to form an RNA stem structure and there is no specific requirement for any of the sequence content of either element for FGFR2 splicing regulation.

*Sequences in a Putative Loop between ISE-2 and ISAR Are Not Required for Splicing Regulation*—A number of RNA stem structures have been shown to function by presenting loop sequences in a single-stranded conformation that facilitates recognition of the loop sequences by RNA-binding proteins. For example, the U1A protein binds to a conserved loop sequence in



**FIG. 6. Deleting all FGFR2 intron 8 sequences between ISE-2 and ISAR does not impair splicing regulation.** The numbers in parentheses in the deletion constructs (13 and 17) indicates the size of the resulting loop between ISE-2 and ISAR compared with a 742-nucleotide loop in pI-11-PANC-W/W37. Abbreviations and lane designations are as described in the legend to Fig. 3.

stem-loop II of the U1 snRNA (51). While such loop sequences are generally present in much shorter loops than the ones proposed here, we sought to determine whether any of the sequences between ISE-2 and ISAR are required for cell specific activation of exon IIIb splicing. The ability to introduce unrelated restriction sites downstream of ISE-2 and upstream of ISAR suggests that FGFR2 loop sequences do not need to be immediately adjacent to the proposed stem. It was previously shown that a deletion of 611 of the 736 nucleotides between ISE-2 and ISAR can be deleted without loss of splicing regulation (Fig. 1, non-regulatory RNA) (40). We made a deletion from the *Asc*I site to the *Not*I site of pI-11-PANC W/W37 that removes essentially all FGFR2 intron 8 sequences between ISE-2 and ISAR. The resulting minigene, pI-11-PANC-del-AN (13), contains 13 nucleotides between the base-paired regions of ISE-2 and ISAR of which 12 are derived from the *Asc*I and *Not*I restriction sites. Transfection of this minigene results in cell-type-specific inclusion of exon IIIb in DT3 cells (Fig. 6, lanes 4–6). We also inserted 4 nucleotides that increased the size of the loop from 13 to 17 nucleotides in minigene pI-11-PANC-del-AN (17) and still observed maintenance of splicing regulation (Fig. 6A, lanes 7–9). Although a single nonbase-paired A residue at the end of ISE-2 is present in the loops predicted in these constructs, we conclude that the length and sequence of FGFR2 sequences that comprise the loop between ISE-2 and ISAR are not critical in cell-type specific activation of exon IIIb splicing.

#### DISCUSSION

In this report we have investigated in detail the sequence and structural components of two intronic *cis*-elements, ISE-2 and ISAR, which are required for splicing regulation of rat FGFR2 transcripts. Deletion or mutation of either ISE-2 or ISAR results in a significant decrease in exon IIIb inclusion

and an increase in exon IIIc inclusion in DT3 cells, suggesting that each element is involved in cell-type-specific activation of exon IIIb splicing and repression of exon IIIc splicing (40, 46). On the other hand deletion of either element has no effect on cell-type-specific exon IIIc inclusion in AT3 cells or other cells that express FGFR2-IIIc. Thus, it has become evident that these elements are required for splicing regulation only in cells that express FGFR2-IIIb. It has previously been proposed that two equivalent human elements (IAS2 and IAS3) function through formation of an RNA secondary structure consisting of 17–18-base-paired nucleotides from each element (43, 46). We have used phylogenetic comparisons of similar sequences from different species as well as the FGFR1 paralogue of FGFR2 and such analysis further supports the hypothesis that splicing regulation by these elements involves formation of a stem structure. Previous analysis of mutations that would disrupt stems formed by the human IAS2 and IAS3 elements showed that such mutations impaired splicing regulation and complementary mutations that would restore the stems likewise restored splicing regulation (43). However, not all of the complementary mutations restored splicing regulation to the levels achieved with the wild type sequences, leading these authors to suggest that some specific sequences within these elements may also be required for efficient splicing regulation. To systematically determine the degree to which a secondary structure as well as the primary sequence content of each element is required for splicing regulation, we determined the effects of complementary mutations in the rat elements, removing the internal bulged nucleotides, and of replacing both elements with unrelated sequences that would maintain stem formation. We found that removal of the bulged nucleotides from ISAR did not impair splicing regulation. Then, to our initial surprise, we also found that replacing ISE-2 and ISAR with completely unrelated sequences that form a stem of similar length maintained splicing regulation that was equivalent to that seen with the wild-type sequences. In studies of the related human RNA structure formed by IAS2 and IAS3 it was hypothesized that some sequence elements within the elements as well as the RNA structure were required for efficient splicing regulation (43). In contrast, we conclude that the primary sequences of ISE-2 and ISAR are not required for splicing regulation, but rather sequences positioned in their location within the intron influence FGFR2 splicing solely through formation of a secondary structure consisting of 17–20 base-paired nucleotides.

Consideration of possible mechanisms through which ISE-2 and ISAR influence splice site selection needs to be considered in the context of other nonsplice-site *cis*-elements that affect splicing of exons IIIb and IIIc (summarized in Fig. 1). An ESS in exon IIIb as well as an ISS upstream of exon IIIb also reduce the efficiency of exon IIIb splicing (26, 41, 42, 52, 53). It has been shown that hnRNPA1 and polypyrimidine tract-binding protein (PTB) play a role in the splicing repression mediated by the ESS and ISS, respectively. However, the repressive effect of these elements on exon IIIb splicing are seen in cell types that express FGFR2-IIIb or FGFR2-IIIc, and therefore they alone cannot account for cell-type specific differences in splicing. In AT3 cells, it appears that this repression is sufficient to preclude exon IIIb inclusion, whereas in DT3 cells factors that can overcome this repression are able to enforce exon IIIb splicing. This activity in DT3 cells (or other cells that include exon IIIb) involves the participation of four different intronic sequences (ISEs) that are collectively able to overcome exon IIIb splicing repression. These include ISE-1 (IAS1), ISE-2 (IAS2), ISAR (IAS3) and ISE-3. In human FGFR2, IAS1 is an ~20 nucleotide U-rich sequence located almost immediately downstream of the exon IIIb 5'-splice site and the protein TIA-1 has been proposed



to increase exon IIIb splicing through binding to this sequence (29). However, TIA-1 is not tissue specific and, depending on its sequence context, IAS-1 can activate splicing of an upstream exon in cell types that express either FGFR2 isoform. A rat sequence we call ISE-1 is a much longer 45 nucleotide U-rich sequence and has also been shown to activate exon IIIb splicing.<sup>2</sup> In addition to ISE-2 and ISAR, we also implicate a role of a fourth additional element, ISE-3, located downstream of ISAR that also functions in activating exon IIIb splicing (40). Breathnach and co-workers (29) have also shown that human sequences downstream of the IAS3 sequences that base pair with IAS2 are also involved in regulation, but include these sequences in the element they refer to as IAS3. In rat FGFR2 transcripts, we have shown that distinct sequences downstream, but not contiguous with ISAR are required for efficient exon IIIb inclusion.<sup>2</sup> Therefore, we refer to such sequences as a separate ISE-3 element. In addition to activating exon IIIb splicing, ISE-2 and ISAR have also been shown to repress splicing of exon IIIc (40, 46). The ability to repress exon IIIc splicing in DT3 cells may be assisted by ESS elements in exon IIIc that are also bound by PTB (45). There is currently no evidence that ISE-2, ISAR, or ISE-3 influence splicing in AT3 (or HeLa) cells regardless of context and thus it is possible that in DT3 cells a cell-type specific factor that interacts with one of these elements may "tip the balance" toward exon IIIb inclusion and exon IIIc skipping (29).

The finding that specific sequences in ISE-2 and ISAR are not required for function other than to generate a double-stranded RNA stem structure has implications for the mechanism through which this secondary structure influences splicing. Numerous studies have proposed models through which RNA secondary structures may be involved in constitutive and alternative splicing. Models have been proposed in which RNA secondary structures that encompass 3'- or 5'-splice sites block recognition of these sites for splicing (54–66). It has also been suggested that secondary structures can influence splice site selection by approximating splice signals across introns (64, 67, 68). In these models the secondary structures themselves are not necessarily bound by proteins that influence splicing, but may simply determine the efficiency with which the constitutive splicing apparatus is able to recognize splice sites in the vicinity of the structure. RNA secondary structures can also influence splicing by facilitating recognition of single stranded RNA sequences in loops predicted to form within the structure (69, 70). Thus far, however, the functions of splicing regulatory proteins have not been shown to involve specific recognition of double-stranded RNA sequences contained within such structures. However, the possibility that RNA-binding proteins can recognize specific bases within a stem was shown for the *Drosophila* B52 protein. Involvement of B52 in regulated splicing is suggested by several studies, although the *in vivo* targets have not been elucidated (71, 72). However, using Systematic Evolution of Ligands by Systematic Enrichment (SELEX), high affinity targets of B52 were identified that were predicted to form short conserved stem-loop structures (71). In this case primary sequences in the loop as well as specific sequences in a predicted stem were both required for efficient binding by B52. Thus, there is a precedent for the possibility that binding of target RNAs by RNA-binding proteins can involve recognition of base-paired sequences within secondary structures.

The secondary structure formed by ISE-2 and ISAR is distinct from other structures suggested to influence splicing in several ways. The most obvious difference is the large distance (736 nt) that separates the ISE-2 and ISAR elements that form

the stem. The ability of an artificial stem structure to sequester and block splicing of an exon located between them was determined (73, 74). In these experiments the complementary sequences were separated by 285 nucleotides and repression of splicing to an exon located between the elements *in vivo* was only seen when the predicted stem structures were at least 50 nucleotides in length. The results suggested that stems separated by this distance either do not form with shorter stems or are not sufficiently stable to influence splice site selection. Experiments using shorter artificial stems to block recognition of a 5'-splice site suggested that RNA structures do not generally form if the loop between them is significantly greater than 50 nt (57). A possible explanation for such a limit in loop size was that following transcription there is a limited "window" during which transcribed RNA exists in a naked form before being bound by hnRNP proteins that would preclude formation of such RNA stem structures (57). The ability of ISE-2 and ISAR to form a secondary structure despite containing 18 base-paired nucleotides located far apart from one another in the intron could be accounted for by the function of proteins that facilitate and stabilize the interaction of these elements. Thus, one possible mechanism suggested that proteins that first bind independently to ISE-2 (IAS2) and ISAR (IAS3) are able to interact with each other and then promote formation of the secondary structure (43). However, based on the findings presented here showing that the specific sequences of ISE-2 and ISAR are not required for splicing regulation, this appears less likely since sequence-specific recognition of ISE-2 or ISAR would be central to this hypothesis. Therefore, as an alternative, proteins bound either upstream of ISE-2 and/or downstream of ISAR may facilitate the approximation of these elements. Likely candidate proteins to perform such a function would include members of the hnRNP family. Packaged mRNP is a dynamic structure and the possibility exists that protein-protein interactions between hnRNP proteins positioned at a distance along a transcript may themselves approximate different regions of pre-mRNAs. Thus, although hnRNP may indeed often prevent RNA structures from forming (57), in selected cases they may facilitate the formation of certain RNA structures.

How does a stem structure formed between ISE-2 and ISAR function to regulate splicing? One possibility is that the stem merely functions to approximate other elements; for example by bringing ISE-3 closer to ISE-1 and exon IIIb. However, when such approximation was achieved by deleting intron sequences from the 5' boundary of IAS3 to a position immediately upstream of and including IAS2, exon IIIb splicing activation was still impaired (43). Similarly removing most of the sequence between rat ISE-2 and ISAR as well as ISAR does not maintain splicing regulation (40). Another mechanism through which the structure might regulate splicing would be through direct binding of a regulatory protein to the double-stranded stems formed between ISE-2 and ISAR. An expanding number of RNA-binding proteins that interact specifically with double-stranded RNA (dsRNA) continue to be described (75). These RNA-binding proteins contain a conserved core of 65–75 amino acids that comprise a conserved double-stranded RNA binding domain (dsRBD) (75). Thus far, the interactions of RNA-binding proteins that contain the dsRBD with target RNAs appear to be sequence-independent and only require a dsRNA of sufficient length for binding (76–80). A crystal structure of the second dsRBD of *X. laevis* X1rbpa has provided information regarding the components of the domain and its RNA target that promote high affinity binding (80). This study determined that a majority of interactions of the dsRBD with RNA involve the phosphodiester backbone and 2'-OH groups, accounting for

<sup>2</sup> R. Hovhannisyan and R. P. Carstens, unpublished results.



sequence-independent binding. Furthermore a 16-base pair dsRNA appears to be sufficient to promote binding. Thus, if in fact the role of the secondary structure formed by ISE-2 and ISAR is, in fact, mediated through binding of a dsRNA-binding protein it is perhaps not so surprising that the specific sequences comprising the stem are not important. The length of the ISE-2/ISAR stem would also be consistent with binding by a dsRBD-containing protein. A number of dsRBD containing proteins have been shown to interact with specific RNAs *in vivo*. For example the *Drosophila* Stauf protein interacts specifically with 3'-UTR sequences of *bicoid* and *oskar* mRNAs and directs their localization (81). However, an unanswered question is how dsRNA-binding proteins can recognize specific transcripts given the sequence-independent binding of the dsRBD. If a specific dsRNA-binding protein is involved in FGFR2 splicing regulation through binding to ISE-2 and ISAR, it is possible that other domains of the protein recognize other intron 8 sequences or structures. Alternatively, an RNA-binding protein (or proteins) bound to specific sequences adjacent to the structure (for example ISE-3) could promote cooperative binding of such a protein to an RNA stem structure through specific protein-protein interactions.

Removing the nucleotides from ISAR that form a bulge in the secondary structure does not impair splicing regulation, and the nonsequence-specific elements tested were also uninterrupted stem structures that were essentially equivalent to the wild-type structure in their ability to maintain splicing regulation. However, all of the predicted wild type structures presented (Fig. 2, B and C) contain either a central bulge or internal loop. Thus, it might be asked why these conserved elements do not also predict uninterrupted stems. One potential answer to this question can be provided by using an analogy to the interaction of dsRNA with the dsRBD of X1rbp. In this structure, the central nucleotides of the dsRNA binding site are not contacted by the protein (80). Furthermore, natural substrates of dsRBDs have been described in which duplex regions are separated by non-Watson Crick base-paired nucleotides (82). Therefore, if in fact the ISE-2/ISAR structure is bound by a dsRBD-containing protein, the bulges may not be necessary for function, but there also may not have been selective pressures to maintain base-pairing in the central region of the stem.

In conclusion, these studies provide further support of the hypothesis that the rat ISE-2 and ISAR elements, like their IAS2 and IAS3 human counterparts, regulate splicing through formation of base pairing interactions between these elements. We have also found that the minimal components of these elements involve only the 18 nucleotides that form base pairing interactions with one another. However, splicing regulation is also maintained when either element is replaced by a completely unrelated sequence provided that a complementary sequence is provided in place of the other element. To our knowledge, this is the first study to demonstrate the function of a naturally occurring RNA *cis*-element involved in regulated splicing that involves an RNA structure independent of any specific nucleotide sequences. Taken together these findings suggest a model in which one *trans*-acting protein component required for FGFR2 splicing regulation is a double-stranded RNA-binding protein. Although numerous RNA-binding proteins have been shown to be involved in regulated splicing, to date a role for a specific double-stranded RNA-binding protein in this process has not been shown. Further identification of proteins that interact with the structure formed by these elements will be required in order to further determine the molecular mechanism through which cell-type specific splicing of FGFR2 is achieved.

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